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FILTERABLE VIRUSES

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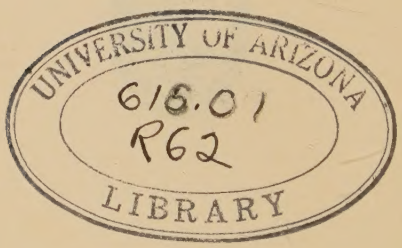

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To the memory of

CHARLES E. SIMON

in evidence of appreciation of his leadership and foresight in the development in the United States of the first Department of Filterable Viruses

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CONTENTS

CHAPTER PAGE

PREFACE ix

I. SOME GENERAL ASPECTS OF FILTERABLE VIRUSES 3
Thomas M. Rivers, M. D., Member of The Rockefeller Institute for Medical Research, New York.

II. FILTERS AND FILTRATION. (1 text-figure.) 55
Stuart Mudd, M. D., Assistant Professor of Experimental Pathology, School of Medicine, Associate in Pathology, Henry Phipps Institute, University of Pennsylvania, Philadelphia.

III. TISSUE CULTURES IN THE STUDY OF VIRUSES 97
Alexis Carrel, M. D., Member of The Rockefeller Institute for Medical Research, New York.

IV. INTRACELLULAR PATHOLOGY IN VIRUS DISEASES. (1 plate and 25 text-figures.) 113
Edmund V. Cowdry, Ph. D., Associate Member of The Rockefeller Institute for Medical Research, New York.

V. VIRUS DISEASES OF MAN AS EXEMPLIFIED BY POLIOMYELITIS 159
Harold L. Amoss, M. D., Associate Professor of Medicine, Johns Hopkins Medical School, Baltimore.

VI. VIRUS DISEASES OF MAMMALS AS EXEMPLIFIED BY FOOT-AND-MOUTH DISEASE AND VESICULAR STOMATITIS 205
Peter K. Olitsky, M. D., Associate Member of The Rockefeller Institute for Medical Research, New York.

VII. VIRUS DISEASES OF FOWLS AS EXEMPLIFIED BY CONTAGIOUS EPITHELIOMA (FOWL-POX) OF CHICKENS AND PIGEONS. (3 plates.) .. 235
Ernest W. Goodpasture, M. D., Professor of Pathology, Vanderbilt School of Medicine, Nashville.

VIII. VIRUS DISEASES OF INSECTS.
Sacbrood of honey bees. (3 plates and 1 text-figure.) 279
The polyhedral diseases. (3 plates.) 301
Rudolf W. Glaser, Sc. D., Associate Member of The Rockefeller Institute for Medical Research, Princeton.

IX. VIRUS DISEASES OF PLANTS. (3 plates.) 335
Louis O. Kunkel, Ph. D., Boyce Thompson Institute for Plant Research, Yonkers.

X. VIRUS DISEASES OF BACTERIA—BACTERIOPHAGY. (2 plates.) 373
Jacques J. Bronfenbrenner, Ph. D., Associate Member of The Rockefeller Institute for Medical Research, New York.

INDEX 419

PREFACE

Although knowledge concerning the exact nature of filterable viruses is lacking, the idea is gaining favor that these infectious agents present many problems which warrant special consideration. An expression of this idea is reflected in the fact that investigators in certain universities are realizing that these disease-producing agents deserve more consideration from bacteriologists and pathologists than they now receive, and that separate departments should be established for their study and for the instruction of students.

Because of the increasing interest in filterable viruses as a group of infectious agents which are worthy of special attention, it is highly desirable to present in a systematic manner some of the chief problems encountered in this field of work. To discuss individually all of the active agents placed in the group by various workers seems injudicious. Therefore, some general aspects of the problems will be considered, and then certain diseases in the different realms of animal and plant life which are thought to be caused by viruses will be discussed. To aid the student and investigator bibliographies comprising representative articles in the various fields of virus work are included with each chapter.

Numerous divergent opinions exist regarding filterable viruses and the diseases induced by them. Because of inadequate evidence many of the opinions have not been generally accepted and it is impossible to state which will eventually be proved correct. For this reason, in the choice of men to coöperate in the preparation of the book, it was thought not only just but proper to select authors with different opinions; each one is entirely responsible for the facts and ideas appearing in his section. Although the ideas in one chapter may not always be in agreement with those in another, the book as a whole reflects precisely the state of affairs as it now exists in the field of filterable viruses.

I am deeply indebted to the authors and to Mrs. C. K. Thompson for their coöperation and assistance in the preparation of the book.

Thomas M. Rivers.

Hospital of The Rockefeller Institute
for Medical Research, New York, 1928.

SOME GENERAL ASPECTS OF FILTERABLE VIRUSES

THOMAS M. RIVERS

CHAPTER I

SOME GENERAL ASPECTS OF FILTERABLE VIRUSES[1]

In all fields of work, times come when investigators must stop and take thought. New facts, new ideas, and new suggestions alter lines of endeavor in every field of research. In view of the recent research activities in the field of so-called filterable viruses, it seems advisable, before entering upon new investigations, to take thought of the knowledge already obtained in regard to these active agents and the diseases caused by them. It is quite obvious that a complete analysis of all work in this field cannot be made in the space allotted a general survey. Therefore, some facts and ideas concerning this group of diseases as a whole will be reviewed briefly and then a few reports concerning several of its individual members will be discussed.

In the accompanying table are listed most of the diseases which are included by different observers in the group under discussion. The etiological agents concerned in these diseases, or groups of them, have been given a variety of names, *e. g.*, filterable viruses, invisible microbes, ultramicroscopic viruses, inframicrobes, protista, microplasms, chlamydozoa, and strongyloplasms. A superficial examination alone is convincing that none of these names is applicable to all of the etiological agents. Names, however, facilitate the interchange of facts and ideas

[1] A paper, Filterable viruses: A critical review, was read by the author before the Society of American Bacteriologists, December 29, 1926, and has appeared in *Jour. Bact.*, 1927, xiv, 217. The editor of the Journal has permitted the author to use the paper, with a few revisions, as Chapter I of this book.

To designate the active agents discussed in the book the term "filterable viruses" was chosen from a long list of unsatisfactory names. For many reasons the term "viruses" is preferable to "filterable viruses," and throughout the book the two will be used interchangeably.

Neither *filterable* nor *filtrable* is in the unabridged dictionaries consulted. The former, however, appears in Funk and Wagnalls' Desk Standard Dictionary, 1927, and the latter in Dorland's American Illustrated Medical Dictionary, tenth edition, 1921. The stem of the verb *filter* is *filtr*. Thus the adjective derived from it would ordinarily be *filtrable*. Nevertheless, in view of the wide usage of *filterable* for many years, particularly by workers in the virus field, it seems advisable to continue the use of *filterable* in preference to *filtrable*.

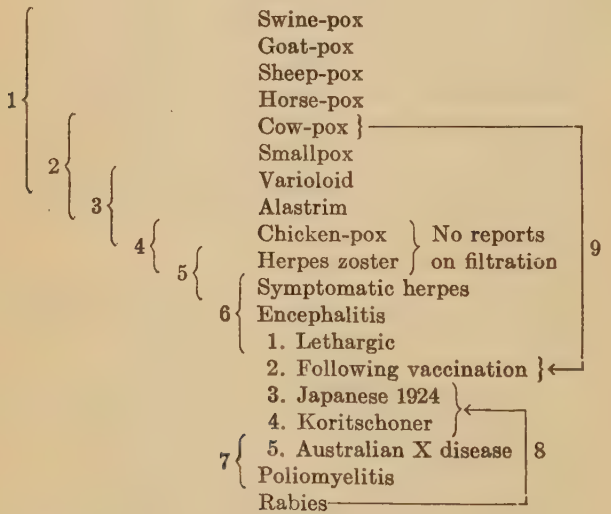
MAJORITY OF THE DISEASES WHICH HAVE BEEN PLACED IN THE FILTERABLE VIRUS GROUP BY DIFFERENT WORKERS

Bacteriophagy

Mosaic diseases of plants (infectious chloroses)

Sacbrood
Wilt of European nun moth
Wilt of gipsy-moth caterpillar
Jaundice of silkworms

Epizoötic of guinea pigs
Hog cholera
Cattle plague (Rinderpest)
Pernicious anemia of horses
Salivary-gland disease of guinea pigs
Virus III infection of rabbits
Foot-and-mouth disease
1. Type A
2. Type O
Vesicular stomatitis of horses
Paravaccinia (No report on filtration)



Borna disease
Fowl plague and plague of blackbirds
Guinea-pig paralysis

Distemper of dogs
Infectious papular stomatitis of cattle
Molluscum contagiosum
Warts
Contagious epithelioma (fowl-pox)
1. Chickens
2. Pigeons
Infectious myxomatosis of rabbits
Rous sarcoma of chickens
Leukemia of chickens
Lymphocystic disease of fish
Epithelioma of fish
Carp-pox
} No reports on filtration

Trachoma* and inclusion blenorrhea
Mumps (According to Kermorgant, a spirochetal disease)
Agalactia (According to Bridré, a bacterial disease)

Measles (rubeola)
German measles (rubella) (No report on filtration)

Grippe (influenza) (According to Olitsky and Gates, a bacterial disease)
Common colds

A. Insect-borne
- a.
  - Nairobi disease of sheep
  - Catarrhal fever (blue tongue) of sheep
  - African horse sickness
  - Pappataci fever
  - Dengue fever
  - Yellow fever (According to Noguchi, a spirochetal disease)
- b. Rickettsia diseases
  - Typhus fever
  - Trench fever
  - Rocky Mountain spotted fever
  - Heartwater disease
  - Flood fever of Japan

B. Bacterial diseases
- Oroya fever and verruga peruviana
- Pleuropneumonia
- Avian diphtheria
- Scarlet fever

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* According to Dr. Noguchi, trachoma of American Indians is a bacterial disease.

between individuals. For practical purposes, then, the term "filterable viruses," mainly because of its wide usage, is as satisfactory as any name suggested. Throughout this discussion the term "filterable viruses" will be employed in a noncommittal way to designate certain active transmissible agents which are capable of producing pathological conditions in bacteria, plants, insects, fish, birds, and mammals, and which by general consent are more or less limited for the moment to the etiological agents of the diseases listed in the table.

The arrangement of the diseases in the table is for convenience of discussion and carries no taxonomic significance. In the first place, filterability of the etiological agents does not sharply delimit this group of diseases, as it is well known that the viruses share this characteristic with certain small bacteria and vibrios, and also with some spirochetes and protozoa. Furthermore, in regard to the etiological agents of some of the diseases within the group, *e. g.*, chicken-pox and paravaccinia, no filtration experiments have been recorded. The viruses of still other diseases within the group, *e. g.*, typhus fever and vaccinia, are either not filterable or filterable with the greatest difficulty. In the past all attempts to classify these diseases have been unsuccessful and there is every reason to believe that such attempts are still premature.

The diseases listed do not form a homogeneous group and some of them should be omitted. The evidence that epizoötic of guinea pigs is a virus disease is not convincing. The Rickettsia diseases do not belong here and there is considerable doubt as to how long the other insect-borne diseases will remain on the list. Most observers no longer consider scarlet fever a virus disease. M'Fadyean, as early as 1908, suggested that pleuropneumonia be classified with bacterial diseases. Oroya fever and fowl diphtheria, in view of Noguchi's and Bordet's experiments respectively, do not belong here. Enough has been said to convince one of the heterogeneity of the diseases listed in the table. In fact they exhibit so many differences that a discussion of the filterable viruses almost amounts to a separate discussion of each disease. Such a state of affairs is due to the fact that the filterable virus group has been used to a considerable extent for the indiscriminate segregation of infectious diseases of unknown etiology. Therefore, it is not unlikely that some of them will be shown to be caused by small bacteria or protozoa. When this occurs, such diseases should be removed from the filterable virus group and given their correct position in the classification of diseases.

Epidemiology

From the epidemiological[2] standpoint the group of filterable virus diseases is notable chiefly because of the remarkable differences exhibited by its members when compared one with another. Some are extremely contagious, *e. g.*, smallpox; others, although inoculable, are not spread by ordinary contact, *e. g.*, rabies; others are insect-borne, *e. g.*, dengue fever and yellows of asters; and still others are transmitted only by grafting, *e. g.*, certain infectious chloroses of plants (Baur). In general, however, the epidemiological problems presented by the virus diseases in regard to regional distribution, seasonal variation, host susceptibility, and virulence are similar to the problems found in connection with other infections.

Immunity

Since immunity, either natural or acquired, plays such an important rôle in all epidemiological studies, a discussion of it at this point is not out of place. With a few exceptions, recovery from diseases produced by filterable viruses is followed by a lasting immunity. In this respect virus diseases differ from those caused by ordinary bacteria. This is not universally true, however, since one attack of typhoid fever produces in the recovered individual a fairly lasting immunity. Many questions have arisen in regard to this kind of immunity, but so far they have not been satisfactorily answered. Nevertheless, a few of the possibilities will be discussed.

Individuals who had measles and chicken-pox in infancy are usually insusceptible to these diseases throughout life. Some investigators state that the serum of these refractory individuals is slightly protective even after a period of 40 years, or contend that their cells are still supplied with sessile antibodies or an increased ability to make them. The majority of the cells in an adult, however, are not the same cells possessed by that individual when 1 year of age. If it is a question of sessile antibodies, then one must suppose that these characteristics are

[2] Epidemic (ἐπί on + δῆμος people) = a disease attacking many persons at the same time.
Epizoötic (ἐπί on + ζῷον animal) = a disease attacking many animals at the same time.
Epiphytotic (ἐπί on + φυτον plant) = a disease attacking many plants at the same time.
In this paper "epidemic" is used in a general way to signify mass disease in bacteria, plants, insects, fish, birds, and mammals.

passed on to daughter cells. On the other hand, a child born of immune parents becomes susceptible to measles and chicken-pox within a few months after birth. Is it possible, then, that the lasting immunity is due to repeated infections so mild that they attract no attention except in the first instance? In regard to diseases as prevalent as measles and chicken-pox this might serve as an explanation. On the other hand, one can hardly explain the persistent protection against poliomyelitis and smallpox upon such grounds.

Another possible explanation for the lasting immunity is that it is due to a prolonged sojourn of the virus in the body or perhaps to its persistence in an individual once infected. Winkler, in his review of immunity to vaccine virus, suggests this possibility. When confronted with the idea one invariably says that it is impossible, because, if it were true, every one would spread measles and chicken-pox. This would not necessarily be the case, however. Typhoid bacilli have been found in the walls of gall bladders many years after attacks of typhoid fever. Furthermore, when the mucous membranes lining the gall bladders are normal, there is little danger of the disease being spread by these carriers. Most human beings carry tubercle bacilli, but only a few spread tuberculosis. Individuals harboring *Treponema pallidum* are not always infectious, particularly in the latent stages of syphilis. Furthermore, syphilis is an excellent example of a disease in which there is a persistent infection coincident with a refractory state in the host to reinfection. For information concerning discussions of this paradox one should read Chesney's review, "Immunity in syphilis."[3]

Now, in regard to virus diseases, is there any evidence (1) of a prolonged or persistent infection, (2) of a coëxistence of infection and refractory state in the host to reinfection from without, and (3) of a causal relation between the prolonged or persistent infection and the lasting immunity? There is considerable evidence that a prolonged infection occurs in some virus diseases and also that this infection can persist for a long time in a host refractory to reinfection. De Kock found a horse's blood infectious 7 years after an attack of pernicious anemia. Sir Arnold Theiler speaks of the persistent infectious nature of the blood of horses that have recovered from pernicious anemia. He also states that the blood of horses inoculated with African horse sickness may remain infective for periods up to 3 months after inoculation. In most instances plants once infected with mosaic virus are never free from it; when they do recover, however, they are not immune. Vaccine virus

[3] *Medicine*, 1926, v, 463.

has been recovered from the lymph nodes of an animal 28 days after inoculation and 22 days after the animal's skin was refractory to reinfection. A prolonged infection occurs in contagious epithelioma, and in regard to this disease Lipschütz says, "Der immune Organismus ist Parasitenträger." Furthermore, Cole and Kuttner have shown that the "salivary-gland virus" of guinea pigs can be obtained at will from immune animals, and in this particular instance it appears that a pig once infected continues to carry the virus indefinitely in spite of a refractory state to reinfection from without. The question as to whether the lasting immunity is dependent *per se* upon a prolonged sojourn or persistence of viruses in the body cannot be answered at the present time. Nevertheless, such an idea, novel in regard to virus diseases, is worthy of serious consideration.

Another interesting feature concerning the immunity to virus diseases is the fact that only active virus protects against a second inoculation of the same virus. In other words, it is doubtful, with a few exceptions, whether injection of a virus completely inactivated leads to a protection against the same virus in an active state. Furthermore, virucidal properties do not appear in the serum of naturally resistant animals which have received repeated injections of active virus. Therefore, it seems that an actively acquired immunity and evidences in the serum of such an immunity are dependent in some way upon an actual infection with the virus, even though it be so mild at times as to give rise to no symptoms.

The degree of active immunity usually exhibited by individuals recovered from virus diseases seems disproportionate to the amount of passive protection afforded by their sera. This fact has led many observers to believe that the protection against virus diseases is predominantly a tissue immunity rather than a humoral one. Be that as it may, protective substances do occur in the sera of individuals who have recovered from certain virus diseases. This is particularly true when animals have been hyperimmunized by means of repeated injections of the viruses, as is evidenced by Gordon's work concerning immunity to vaccinia.

Virucidal properties, precipitins, and complement-fixing antibodies have been demonstrated in the sera of individuals who have recovered from certain of the virus diseases. Gordon and others believe that agglutinins exist in the sera of animals that have recovered from vaccinia, inasmuch as "vaccine granules" are agglutinated by such sera. Until these granules have been definitely shown to represent only

vaccine virus, one must look upon the supposed agglutinins with a great deal of suspicion.

Prevention of Diseases Caused by Viruses

The prevention of diseases caused by viruses depends upon the protection of susceptibles from exposure or upon their immunization. Quarantine measures are established for protection, but frequently they are ineffectual. The eradication of insects or protection against them controls the spread of insect-borne diseases. Furthermore, susceptible species can be replaced by naturally insusceptible ones, and at times it is possible to breed refractory hybrids. This method has been used advantageously where animals and plants are concerned. Unfortunately, however, such methods cannot be employed in dealing with all animal diseases, nor can they ever be used in dealing with human diseases. Under these conditions attempts are made to decrease the number of susceptibles by means of vaccination with attenuated or modified viruses.

Filterability

Since the discovery of the first filterable virus in 1892, it has been determined by means of different kinds of filters that many diseases are caused by active agents smaller than ordinary bacteria. Some are presumably much smaller and are most likely optically immeasurable. Others, however, do not seem to be so small and concerning the filterability of these there is much discussion. Methods of filtration are crude and inaccurate and the most one can say regarding the viruses is that under given experimental conditions they either pass or do not pass through certain filters. The failure to pass through a filter, however, is certainly not determined in every instance by the size of the virus. The electrical charge on the virus, the electrical charge on the filter, the adsorption of the virus by aggregates of protein or by cell detritus, the amount of protein or other substances in the virus emulsion, the temperature at which the filtration is conducted, the amount of negative or positive pressure employed, the duration of filtration, and other factors not mentioned or not known serve to influence the results of all filtration experiments. Furthermore, sufficient attention has not been given to the possibility that some filters may not only hold back certain viruses but may also inactivate them in some manner so that they can never become active again.

Filters, in spite of their faults, are useful in working with diseases of

unknown etiology, and by means of them one is able at times to determine quickly whether a given disease is produced by an agent smaller than ordinary bacteria. Sometimes, however, small bacteria may still contaminate the filtrates or two viruses may be present in the filtered material. Therefore, filtrates from uncontrolled, and even from well-controlled sources may contain more than one active agent, some of which may be cultivated on simple or on special media. All investigators should be extremely careful in working with filtrates not to be misled by their findings and ascribe to an active agent an etiological rôle in a disease with which it has nothing more than accidental connection.

Size

Very little has been recorded in regard to the size of many of the viruses other than that they pass through certain kinds of filters. It is obvious that this method only indicates roughly that the viruses which pass through tight Chamberland candles are very small. No virus has been obtained in an absolutely pure state. Not even the washed granules of vaccine virus can be accepted as representing only virus. Therefore, it is impossible to say that virus alone is being filtered rather than virus attached to aggregates of protein or particles of degraded cells. Nevertheless, attempts have been made in various ways to determine the size of a few viruses. According to d'Herelle, the diameter of the phage is 20–30 $\mu\mu$. Bechhold and Villa state that its diameter is > 35 $\mu\mu$ and < 200 $\mu\mu$. Duggar and Karrer believe that the virus of tobacco mosaic is approximately the same size as the colloidal particles of fresh 1 per cent hemoglobin, 30 $\mu\mu$. Zinsser and Tang are of the opinion that bacteriophage, Rous' sarcoma virus, and herpes virus are > 20 mμ[4] and < 100 mμ in diameter.

The size and weight of molecules of crystalline egg albumen and crystalline hemoglobin are not agreed upon. Bechhold states that an aggregate of 50 molecules of egg albumen is > 4 $\mu\mu$ and < 10 $\mu\mu$ in diameter. According to du Noüy, however, 1 molecule of egg albumen is 4.1 $\mu\mu$ in diameter. If it is difficult to determine the size of molecules of relatively pure crystalline substances, what hope is there at present of ascertaining the size of the viruses which have not been obtained in a

[4] $\mu\mu$ and mμ have usually been employed interchangeably to represent a millimicron, the thousandth part of a micron or the millionth part of a millimeter. According to Uhler (*Science*, 1927, lxv, 232), these symbols are not interchangeable; mμ represents a millimicron (10^{-6} mm.) and $\mu\mu$ is the symbol for micromicron (10^{-9} mm.). Nevertheless, in the present book, both symbols represent 10^{-6} mm.

pure state? Furthermore, it is useless to pretend to know what is the lower limit in point of size for living things. In general, however, it can be said that many viruses are probably of sufficient size to exist in a living state, and that others are probably small enough to satisfy the demands of those who insist that they are not possessed of life.

Cultivation

Following the discovery of the first filterable virus, 36 years ago, numerous workers have claimed to have successfully cultivated *in vitro*, by means of simple or complex media, one or more of these active agents. Since all claims cannot be discussed, only the outstanding ones will be considered. The term *in vitro* will be avoided as there seems to be no agreement as to its exact meaning. Therefore, the cultivation of viruses in the presence or absence of living cells will be discussed.

There is no reason to doubt that the viruses causing vaccinia, herpes, typhus fever, Rous' sarcoma, and Rocky Mountain spotted fever have been successfully cultivated in the presence of living cells in tissue cultures. Moreover, Levaditi has stated that the virus of poliomyelitis either survived or multiplied in fresh spinal ganglia (monkey) placed in plasma. Harde was able to grow vaccine virus in the presence of living corneal cells, but, if the cells were killed by freezing or by hypertonic salt solution, the virus failed to multiply. The virus of fowl plague is cited as one that has been cultivated *in vitro*. One might ask, however, if it has been cultivated in the absence of living cells, inasmuch as a large amount of blood was added to the medium employed. Furthermore, Landsteiner and Berliner found that the virus would not multiply if laked or frozen blood was used, and stated in their report that one could not say that growth of the virus had taken place in a lifeless medium. The statement is frequently seen that Bordet cultivated the etiological agent of contagious epithelioma of chickens. Such statements are incorrect. Bordet claims to have cultivated a small bacterium which causes avian diphtheria. Furthermore, he specifically states that the bacterium does not cause contagious epithelioma and that avian diphtheria and contagious epithelioma are two distinct diseases in spite of the view held by some investigators.

There are excellent reasons for stating that the etiological agents of foot-and-mouth disease, rabies, and poliomyelitis have not as yet been cultivated in the absence of living cells. The evidence is insufficient to convince one that "globoid bodies" are etiologically associated with poliomyelitis (Amoss). A consideration of the survival or the multi-

plication of the virus of poliomyelitis in the presence of pieces of fresh kidney is not germane to the discussion. Streptococci have been given an etiological rôle in many virus diseases. It is unlikely, however, that they cause all of the following diseases: poliomyelitis, lethargic encephalitis, influenza, measles, and German measles. Gye's reports concerning the cultivation of the virus of Rous' chicken sarcoma will be discussed further on.

Recently Olitsky reported that he was able to cultivate the virus of tobacco mosaic in a simple medium presumably free of cells. Mulvania and Purdy, working independently, were unable to confirm his work and suggested that his results are open to several interpretations. An observation of the kind reported by Olitsky, if correct, is one of fundamental nature and significance. In fact, it is of almost as much significance as an observation upon the cultivation of bacteriophage in the absence of living bacteria. Unequivocal confirmation of Olitsky's work, therefore, would settle one of the most important problems in the whole field.

In general it can be said that no worker has proved that any of the etiological agents of the diseases in the table, down to trachoma,[5] is susceptible to cultivation in the absence of living cells. A satisfactory explanation of the difficulty experienced in cultivating the viruses on artificial media is not easily found. Their small size alone should not make them insusceptible to cultivation. Nor does it seem to be a question of delicacy or sensitiveness, because many of them are extremely resistant to chemical and physical agents. Therefore, the viruses appear to be obligate parasites in the sense that their reproduction is dependent upon living cells. Whether this reproduction occurs intra- or extracellularly is a debated question.

Cell Types in Relation to Virus Reproduction

In view of the fact that viruses apparently multiply only in the presence of living cells, it is advisable to ascertain what kinds of living cells promote their reproduction best, and what effect upon the cells is induced by this reproduction.

Species Specificity.—A remarkable species specificity is exhibited by many viruses. Rous' sarcoma grows only in chickens. Sanarelli's virus of infectious myxomatosis and Virus III are active only in rabbits.

[5] The report concerning Dr. Noguchi's work on the cultivation of the etiological agent of trachoma appeared in *Jour. Am. Med. Assn.*, 1927, lxxxix, 737.

The "salivary-gland virus" described by Cole and Kuttner apparently affects only guinea pigs. A wilt virus that attacks one kind of caterpillar is innocuous for other caterpillars. The virus of poliomyelitis is active only in man and the monkey.

Importance of Young Cells.—Frequently young cells seem essential for the activity of viruses. The bacteriophage multiplies only in the presence of young bacteria. Old or dead bacteria are not lysed except in the presence of young, growing forms. The activity of mosaic viruses is manifested only in young leaves. This applies also to the infectious chloroses transmitted by grafting only. Virus diseases usually attack insects in certain stages of development. In the higher forms of life virus activity is also best exhibited not in old, undernourished, sickly individuals, but in young, healthy ones. Injury also plays an important rôle in the infectiousness of many virus diseases, not the rôle, however, of furnishing a *nidus* of dead tissue for the growth of the viruses, since dead tissues do not promote their growth, but the rôle of furnishing young cells or growth-promoting factors usually found in their vicinity. Injury necessitates repair. Restoration, as is well known, is accompanied by young cells. In a rabbit's cornea and skin the activity of vaccine virus, herpes virus, and Virus III is first seen along the lines of scarification. Furthermore, the evidence of this activity is first found in the young cells filling in the defects caused by the injury. Old cells may become involved later. Rous and Murphy, working with chicken sarcomas, observed phenomena that seem to be related to injury. Injections of virus emulsion and kieselguhr give rise to more rapidly appearing and more diffusely growing tumors than does the inoculation of virus emulsion alone. More metastases are found in the ovaries during egg-laying seasons than during quiescent periods. These phenomena are probably due to the presence of young cells participating in the repair of injured tissues.

Cytotropic Phenomena.—In some diseases produced by ordinary bacteria and in many caused by viruses, a certain amount of "selective tissue localization" is apparent. Variola, chicken-pox, and contagious epithelioma usually attack the skin; rabies and poliomyelitis, the brain; Rous' sarcoma, mesodermal tissue. Inasmuch as vaccine virus, herpes virus, and chicken-pox virus attack both ectodermal and mesodermal cells, it seems that a position of exaggerated importance has frequently been accorded this phenomenon of "selective tissue localization." Nevertheless, some viruses, *e. g.*, those of rabies and poliomyelitis, exhibit a remarkable affinity for cells of certain tissues and apparently

can neither multiply nor produce signs of disease unless they come into a close relation with these cells.

Injury naturally plays an important part in the infectiousness of diseases that exhibit pronounced cytotropic phenomena. In this case it is not the function, however, of providing young cells, but the rôle of mechanically making it possible for the viruses to come in contact with susceptible types of cells.

Effects of Viruses upon Cells

It has been shown that some viruses multiply only in a restricted number of hosts, that frequently this multiplication occurs only when the virus is in close relation with certain types of cells, and that young, actively growing cells play an important rôle in the infectiousness of many virus diseases. Now a word will be said in regard to the effects produced in cells by viruses. At first the involved cells frequently show a remarkable increase in size and in rate of multiplication and often exhibit amitotic division of the nuclei. The enhancement of size gives one the impression that it is related to growth phenomena and to imbibition of fluid. This process is spoken of as "ballooning degeneration." Eventually the cells die and go to pieces. This process is spoken of as "reticulating colliquation." Two forces seem to operate: one stimulating the cell, the other destroying it. Consequently the picture produced by a virus disease is more or less dependent upon which of these forces predominates. Chicken-pox, foot-and-mouth disease, zoster, variola, and lysis of bacteria by phage are diseases in which destructive agencies predominate. Rous' sarcoma, contagious epithelioma, fowl leukemia, and warts are diseases in which stimulating forces are dominant. A few observers have attempted to classify the cytotropic viruses under cytolytic and cytokinetic headings with subdivisions under each according to the type of cells involved. Such a classification is premature.

It is not known whether the viruses multiply intra- or extracellularly. Nevertheless, they have a profound influence upon cells and cause remarkable changes within them. This influence most likely accounts for the fact that in lesions caused by many viruses intracellular changes assume appearances characteristic enough to be spoken of as inclusion bodies. In this respect many virus diseases differ from those caused by ordinary bacteria.

Inclusion Bodies

Viruses usually produce characteristic macroscopic lesions in plants, insects, birds, and animals, or cause marked changes in their condition. Such alterations and lesions serve as indications of virus activity. In addition to the characteristic macroscopic lesions already mentioned, many viruses also produce equally characteristic microscopic changes as evidenced by the presence of inclusion bodies in the nuclei and cytoplasm of affected cells. There is no obvious reason why these microscopic changes, inclusion bodies, should not be used as guides or indicators with the same degree of readiness as that with which the macroscopic lesions are employed. Diagnoses based upon the pictures presented by aggregates of cells, as in tuberculosis and cancer, are familiar to all and no one is astonished any more at the correct diagnoses made by competent pathologists. Why not, then, use pathological pictures found within cells as aids in diagnostic and experimental work? Intracellular pathology can be used in this manner. As a matter of fact, it has been used for a long time in the diagnosis of rabies and smallpox.

Inclusions have been seen in the cells of plants, insects, fish, birds, and mammals affected by virus diseases. Many of the inclusions described, however, cannot be accepted as specific or characteristic, and it is these that detract from the significance of the ones well established and accepted by numerous critical investigators. No worker familiar with the microscopic pathology of the virus diseases doubts the importance and significance of Guarnieri bodies, Negri bodies, Bollinger bodies, and the nuclear inclusions seen in varicella, herpes, and several other virus diseases.

Various ideas are held concerning inclusions. Some investigators consider them merely as products of degeneration, but others believe that they are the virus itself, while yet others think of them as virus surrounded by a mantle of altered cellular material. As yet their nature has not been definitely determined. Nevertheless, in spite of the ignorance concerning their nature, inclusion bodies have held and will continue to hold an important position in the study of this group of diseases. Many attempts to produce significant inclusions by artificial means have been unsuccessful. Therefore, under properly controlled conditions the presence of inclusions, accepted as significant, will undoubtedly in the majority of instances be indicative of the presence of a virus in the immediate vicinity.

Inclusion bodies have not been found in all virus diseases. In some they may have been overlooked, while in others they may not occur. A restudy of the diseases of unknown etiology may reveal many interesting changes within the cells. These studies, however, must be made or guided by well-trained men with a wide knowledge concerning normal and pathological tissues in order to prevent the literature from being flooded with reports dealing with inclusions not of a characteristic or specific nature.

Resistance to Physical and Chemical Agents

It is generally believed that viruses are more resistant to glycerol than are ordinary bacteria. This is not universally true, however. Virus III, the "salivary-gland virus" of guinea pigs, and others are not active after remaining in 50 per cent glycerol for 6 weeks. On the other hand, many bacteria, particularly if in tissues, remain viable in glycerol much longer than 6 weeks. The extensive use of glycerol for the preservation of viruses is probably largely dependent upon the fact that viruses are usually very susceptible to conditions in autolyzing tissues and that glycerol acts as a desiccant and retards autolysis of the tissues containing the viruses.

All viruses are inactivated by high temperatures. The degree of heat necessary to accomplish this varies from 45° to 80°C., depending on the virus. The majority of them resists low temperatures. The results of repeated freezing and thawing with liquid air (−185°C.), however, do not sharply separate them from ordinary bacteria on the one hand, and from enzymes on the other. Under ordinary conditions some retain their activity *in vitro* for periods of 5 years, others become inactive within 48 hours. Some resist putrefaction and drying. Extraction with chloroform, acetone, alcohol, and toluol for periods of 2–8 days does not inactivate some dried viruses (vaccine virus). A few, even in a wet state, are not inactivated in this manner (virus of tobacco mosaic). Dry spores of certain bacteria (*B. subtilis*) also resist extraction by means of these agents. The virus of contagious epithelioma is active after exposure for 24 hours to 1 per cent sodium hydroxide.[6] Three per cent phenol does not inactivate the virus of African horse sickness. Plant cells are supposed to be more tolerant of the injurious action of bile and saponin than are animal cells. In view of the general susceptibility of the viruses to the action of these agents, some workers are inclined to believe that they are more closely related to protozoa than

[6] According to Friedberger, this statement is incorrect.

to bacteria. There are a number of striking exceptions to the rule, however. More evidence is not needed to convince one that a wide range in the degree of resistance to physical and chemical agents is exhibited by the viruses, and that a classification based upon resistance to such agents is as impossible of accomplishment as is an adequate classification of ordinary bacteria by means of thermal-death-point determinations.

Corpuscular Nature of Viruses

The question of the organized or corpuscular nature of the viruses has not been satisfactorily settled. This is due to the fact that most workers realize that the granules which are seen, which are frequently held back by filters, and which are thrown down by prolonged centrifugation, may not represent virus alone. This is particularly true since viruses exhibit a remarkable tendency to be adsorbed by many things with which they come in contact.

Do Viruses Respire

Very little information concerning the respiration of viruses is available. Recently, however, Bronfenbrenner reported that he was unable to detect any respiration on the part of phage, herpes virus, and rabies virus in the absence of living host cells. He also found, taking the rate of multiplication into consideration, that growing bacteria plus phage show no more respiratory activity than do growing bacteria alone. These facts are interesting, yet they do not conclusively answer all questions concerning the respiration of viruses, inasmuch as Ohga has shown (1) that "Indian lotus fruit which had lain buried in a bed of peat probably for more than 200 years showed about 100 per cent germination when the fruit coats were made permeable to water by a treatment with sulfuric acid," and (2) that "the respiration rate of air-dry fruits containing about 12 per cent of water was so slow that a measurable amount of carbon dioxide was not obtained after three days' continuous absorption in sodium hydroxide."

"Mutation" of Viruses, or the Results of Their Adaptation to New Hosts

Mutations of bacteria with concomitant changes in their characteristics are at present of particular interest to bacteriologists. Naturally, the question arises then as to whether viruses can mutate. In the field of filterable viruses, however, this is not a new question, inasmuch as it has been under discussion in regard to the relation between

vaccine virus and the virus of smallpox since Jenner's time. In spite of all contradictions, it seems that smallpox virus passed through several calves becomes vaccine virus. Furthermore, if a sufficient number of passages is made in calves, it is impossible for this altered virus to regain the characteristics of smallpox virus even after repeated passages in men. Observations of a similar nature have been made in regard to other virus diseases, *e. g.*, contagious epithelioma of chickens and pigeons, and mosaic disease of tobacco and cucumbers. Whether it is correct to speak of these phenomena as examples of mutation is not known. In any event, when viruses are adapted to alien hosts, their characteristics are frequently altered, as well as those of the diseases produced by them.

Do the Viruses Exist in a Living State

The question as to whether the viruses are animate or inanimate is also an old one, inasmuch as it was propounded simultaneously with the discovery of the filterable nature of the viruses. Beijerinck's idea of a living, contagious fluid called forth many protests. Sanfelice working with fowl-pox in 1914, found that the virus was not inactivated by 1 per cent sodium hydroxide, and, because of this fact, he was led to think of it as an inanimate poison capable of attacking normal cells and producing within them a poison of a similar nature which in turn could attack other normal cells. Thus he described his idea of how a lifeless agent might be passed in series reproducing itself indefinitely. The work of Twort, d'Herelle, Bordet, and others, on the bacteriophage, is familiar to all. The numerous discussions concerning the nature of this active agent have led investigators to question more closely the living nature of other filterable viruses. Many tests have been devised to act as criteria for the presence of life, but so far no one of them has been found satisfactory. Therefore, it is impossible at present to say whether the viruses are animate or inanimate. It is wise to leave the subject at this point, as further pursuit of it leads one into the sterile discussion of what life is, a problem still in the realm of metaphysics.

Identity of the Epitheliotropic and Neurotropic Viruses

In the table the diseases from swine-pox through rabies have been arranged and bracketed in a way that quickly shows the relation claimed by different workers to exist between members of the group. There is undoubtedly a close relation between the diseases in the upper part of the group, swine-pox through alastrim. It is now generally believed

that chicken-pox and smallpox are distinct and different diseases. This has not always been the case, however, and as late as the middle of the nineteenth century Hebra taught that they were identical. Even at the present time there is a difference of opinion in regard to the relation of smallpox and varioloid to alastrim on the one hand, and of chicken-pox to alastrim on the other. Gildemeister and Herzberg recently offered experimental evidence to support the idea of a close relation between herpes virus and vaccine virus and suggested that one might be a mutant of the other. Bókay's paper on the relation of chicken-pox to herpes zoster appeared in 1909, and since then a number of other papers have appeared in which the idea that the two diseases are identical has been supported or opposed. For many years there has been much discussion concerning the interrelationship existing between the various kinds of herpes, and this interest has been stimulated by the work of Doerr, Levaditi, Flexner and Amoss, and others on herpes and lethargic encephalitis. There is a great confusion of ideas and facts in regard to encephalitis. Several kinds of viruses have been obtained from the brains of individuals who have died after showing signs of encephalitis, and to each of these viruses has been ascribed an etiological rôle. I am not convinced that this rôle, in many instances, is more than an accidental one. Furthermore, since encephalitis follows a number of infectious diseases, I am not convinced that the brain is the proper place to look for the etiological agent, inasmuch as other agents besides viruses and bacteria attack nervous tissue, toxins for instance.

Many viruses may be closely related or some may have evolved from a common ancestor. Nevertheless, it will be hard to convince observant workers that chicken-pox, symptomatic herpes, and smallpox now possess much in common. Since a great deal of the evidence in favor of the identity of these viruses has been obtained by means of cross-immunity experiments conducted in the skin of human beings and animals, it is possible that a factor generally overlooked or underestimated is responsible for some of the confusion. Jenner and others of his time recognized the fact that skin diseases, exanthems, and extensive mechanical injury might induce a temporary refractory state to vaccine virus. This phenomenon was thought to be dependent upon some non-specific factor. Ledingham recently reported that India ink injected into the skin of a rabbit rendered it resistant locally to vaccine virus for 49 days. Carnot has shown that skin treated several times with ultraviolet light is temporarily refractory to vaccine virus. Experienced workers invariably tell inexperienced ones not to use areas of

skin previously handled when conducting cross-immunity experiments. Busson has reported that guinea pigs immunized a short time previously to vaccine virus resisted a known lethal dose of rabies virus. He thought that the protection was probably non-specific. Enough has been said to show how easily one might be misled in regard to the identity of the viruses if one is not cognizant of the difficulties usually encountered in this field of work.

Malignant Growths

A discussion of malignant growths in connection with filterable virus diseases may seem out of place. Nevertheless, recent reports concerning the nature and origin of tumors make it necessary to say a word in regard to this subject.

Sanarelli, in 1898, described an infectious myxoma of rabbits. Later Moses was able to transmit the tumor by means of sterile filtrates. Rous and Murphy have shown that some spontaneous tumors in chickens are transmissible by means of tumor filtrates. These chicken tumors have led to a great deal of discussion in regard to their importance in the general study of malignant growths. Recently increased interest has been aroused in the Rous sarcoma and in the general cancer problem by Gye, who reported that two factors are essential for the production of malignant growths, one of which is a living, organized virus capable of multiplication in certain complex media. The virus cultivated under these conditions, however, is innocuous, and only becomes capable of producing tumors when mixed with a second specific factor found in tumor filtrates inactivated by means of chloroform, heat, and antiseptics. Murphy, Cori, and Harde are of the opinion that Gye did not properly control his experiments. Furthermore, the two former workers have been able to reactivate a chloroform-treated filtrate by the addition of substances containing no virus. Since Gye has not conclusively demonstrated that all the virus in his treated filtrates was inactivated, and since others have shown that 4 cc. of such filtrates produce tumors when 2 cc. fail, the proof that two factors are essential or that the virus has multiplied in cultures is unconvincing.

Gye's ideas and experimental results concerning the production of chicken sarcomas are diametrically opposed to those of Carrel, who has reported that he was able to produce sarcomas, transmissible by filtrates, by injecting into chickens embryonic tissue mixed with tar, arsenic, or indol. Carrel believes that the etiological agent of these tumors

originates within cells of the host under certain conditions and is of a phage-like nature in the sense that it transforms normal cells into malignant ones which die and liberate more of the active agent. In this manner the virus is supposed to reproduce itself indefinitely. On the other hand, Murphy and Landsteiner were unable to produce sarcomas, transmissible by filtrates, by injecting into chickens mixtures of embryonic tissue and tar. Carrel's conception of the origin of tumors is not a new one, inasmuch as Sanfelice, in 1914, Twort, and Doerr more recently, voiced exactly the same idea. Nevertheless, no one has offered as much evidence as Carrel that the conception is correct. In view of the fundamental significance of such a conception from a broad biological standpoint, reports concerning attempts to confirm this work will be eagerly awaited by workers in this field. At the present time no final conclusion can be drawn.

Future Study of Virus Diseases

The etiological agents of some of the diseases in the table on pp. 4 and 5 will probably be cultivated or otherwise definitely identified. Consequently, attempts to cultivate them should not be abandoned entirely. On the other hand, the indications are that some viruses will not be cultivated in the absence of living cells until cells and their activities can be more accurately imitated. Therefore, cultivation experiments should not engage one's entire attention. Kraus and others have suggested that filterable stages of bacteria, protozoa, and spirochetes may play a rôle in certain virus diseases. This is unlikely. The fact that it is impossible to cultivate in simple media the filterable forms of these etiologic agents does not explain why the non-filterable forms are invisible or insusceptible of cultivation.

How, then, is progress to be made in the study of virus diseases? It will be difficult for the best trained workers and doubly difficult for those poorly trained to progress rapidly in this field. As long as viruses resist cultivation on simple media, just so long will it be necessary to study them in the host, in the tissues of the host, or in emulsions and filtrates of the tissues. At times it may be impracticable to study a disease adequately in its natural host, *e. g.*, man. In such instances efforts are usually made to establish the disease in a suitable experimental host. In order to recognize and identify the disease in the new host, the worker must be familiar with the clinical and pathological picture in the old one and at the same time capable of employing the

necessary immunological tests. Furthermore, it must be remembered that the new host may decidedly alter both the disease picture and the virus, and that this host may be subject also to natural virus diseases of its own. Therefore, under such circumstances, one should be careful in explaining the unknown in the old host by means of facts obtained from studies conducted in the new.

Sufficient attention has not been paid to the effect that viruses have upon each other when acting simultaneously or alternately in the same animal or to the effect that other kinds of diseases have upon the localization and activity of viruses. In regard to herpes zoster, herpes simplex, cancer, and encephalitis following vaccination, one frequently hears the statement that a latent virus has become active in the presence of injury or the activity of a new virus. I have already suggested that some viruses might persist in the body for a long time. In only a few instances, however, is there definite proof of this persistence. In how many more it occurs is not known. One should not assume the presence of a latent virus without at least attempting to prove the validity of the assumption.

Prevention of a virus disease does not necessarily depend upon the visibility of the etiological agent or upon a complete knowledge of its pedigree, as is evidenced by vaccination against smallpox and rabies. It appears that the use of active virus either in small amounts or in an attenuated state is the only means by which a lasting protection can be obtained. Money and time would be well spent, then, in attempting to remove the unnecessary and objectionable ingredients of virus emulsions used for vaccination purposes. Moreover, it might be wise to make further attempts to determine whether, in some instances, instead of vaccination with active viruses, the repeated subcutaneous injections of purified and inactivated viruses lead to a degree of immunity sufficient to warrant their use.

SUMMARY

In the majority of virus diseases a close relationship between the etiological agents and the cells of the hosts exists. This intimate type of parasitism is emphasized by the fact that some of the diseases exhibit a striking species specificity, that the viruses have resisted cultivation in the absence of living cells, that characteristic or specific pathological changes are frequently observed in cells affected by viruses, and, finally, that a host once recovered from a virus disease usually exhibits a lasting immunity.

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FILTERS AND FILTRATION

STUART MUDD

CHAPTER II

FILTERS AND FILTRATION

Porcelain or kieselguhr filters, capable of retaining ordinary bacteria, were introduced in the last decades of the nineteenth century and have since found increasingly widespread use in sterilizing thermolabile materials and in the study of filterable viruses. The use of ultrafilters in certain fields of work assumed significance in the first decade of the present century and has since become an aid in the study of viruses. Asbestos discs and plaster of Paris filters have latterly been introduced for practical and experimental use respectively. An understanding of the process of filtration, however, has by no means kept pace with the manufacture and dissemination of filters. The apparent simplicity of filtration has been a pitfall, leading usually to incomplete recording of essential data and frequently to unwarranted conclusions. In view of the confusion of ideas existing in regard to filters, the author will attempt to describe the common ones, the proper methods of using them, and the conclusions one is justified in making from data obtained by their correct use.

Types of Bacterial Filters[1]

Porcelain Filters.—Bacterial filters were first introduced by Chamberland (46) in 1884. He described his method as "filtration à travers un vase poreux en porcelaine dégourdie, mode de filtration qui est employé dans le laboratoire de M. Pasteur pour séparer les microbes de leurs milieux de culture." The "bougies filtrantes" of Chamberland and Pasteur are manufactured under the name "Filtre Chamberland Système Pasteur," and are referred to in the literature as "Chamberland," "Pasteur," or "Chamberland-Pasteur" filters. They are hollow unglazed candles, closed at one end, which are made from finely ground kaolin (hydrous aluminum silicate) with the addition of quartz sand (silica), and burned at a temperature just low enough to avoid sintering (148). Such bacterial filters, in addition to the Chamberland, have been sold by a number of porcelain manufacturers; occasional references

[1] Rosenthal (148) has described the different types of filters, accessory apparatus used in filtration, and the methods of filtration usually employed.

to them are found in the literature under the names of Doulton (English), Maassen (German), Allen (American), and Coors (American).

The Chamberland filters for laboratory use are now made in graduated porosities described by the manufacturers as follows:

"L 1, the most porous, is designed to let pass all microbes. L 1 bis and L 2 retain coarse microbes and let pass the smallest, such as that of (bovine) pleuropneumonia. L 3 arrests even the bacillus of diphtheria and the spores of tetanus. [Bougies made from] the other pastes are more and more dense and are true filters; they are marked L 5, L 7, L 9, L 11 and L 13 (in order of increasing fineness of pore)." The water filters marked F and B are equivalent in porosity to the laboratory bougies L 5 and L 7 respectively.

Concerning the manufacture and testing of the filters the makers give the following description (160):

"L'homogénéité de la pâte (différence dans sa composition), alliée au degré de cuisson de la porcelaine dans chaque numéro de pâte est la raison primordiale pour la parfaite filtration des liquides.

"L'essai de ces bougies est fait en les trempant dans l'eau, à la pression d'air de un kilo, la bougie ne doit pas laisser passer de bulles d'air, si ce défaut se produit elle est mise au rebut.

"Quant à la dimension des pores, elle est calculée, a l'aide d'une colonne de mercure adoptée à la bougie: on la trempe ainsi armée dans un vase rempli d'eau, le mercure monte dans le tube de verre et suivant la hauteur atteinte, par une formule adéquate, on obtient la dimension des pores de la bougie."

The method described above of testing porosity is Grenet's (68). The details of the computation were not included in Grenet's communication and are now withheld by the manufacturers as a trade secret.

Pukall (143) described filters, usually balloon-shaped, made of a kaolin-quartz mixture and burned at a temperature of 1200°C. or more. Garros (65) manufactured filters of "porcelaine d'amiante, made from finely pulverized asbestos, burned to a porous mass." "Kitasato filters" are slender porcelain candles of German manufacture resembling those used by Kitasato (89) in the study of tetanus toxin. Reichel used apparatus that required filters of special design; these have been made from porcelain and also from kieselguhr.

Kieselguhr Filters.—Observation of the clarity of seepage water in the kieselguhr deposit near Celle, Hanover, in contrast to the discolored ground water in adjacent regions led to the use of diatomaceous earth for water purification. Bacterial filters of kieselguhr (System Nordtmeyer-Berkefeld), similar in design to the Chamberland-Pasteur bougies, were first described in 1891 by Nordtmeyer (132) and Bitter (35).

The manufacturers submit the following account of the manufacture and testing of Berkefeld filters (28):

"The material from which these filters are manufactured is a special kind of 'Kieselgur' (diatomaceous earth, fossil meal) found not far from Celle. The raw material is carefully washed and sifted to free it from sand and other impurities; it is then mixed with asbestos and organic matter, pressed in cylinder form and dried. The dried cylinders are burnt in a kiln at a temperature of about 1200°C. and the burnt material is shaped on special machines into the desired form of hollow cylinders of various sizes. These sizes vary from 25 cm. length by 5 cm. diameter (the ordinary cylinder for house and industrial filters) to 3 cm. length by 1½ cm. diameter (the smallest laboratory filter). The shaped cylinders are thereupon fitted into metal or porcelain headpieces by means of a special cement. This work requires great care and experience, in order to obtain perfectly germproof joints. Each filter is finally tested by blowing compressed air into the cylinder submerged in water. By careful inspection any flaw or fault in the cementing will be detected.

"The grading of the cylinders, N (normal), V (coarse) and W (dense), * corresponds with a scale showing the filtering capacity with pure (filtered) water under a certain pressure.

"The size of the pores is about 3– 4μ in the W grade.
"The size of the pores is about 5– 7μ in the N grade.
"The size of the pores is about 8–12μ in the V grade.

"The substance of the filtering cylinders is purely siliceous, and therefore quite indifferent to water, alcoholic, aetherial or organic liquids."

* W signifies "wenig Wasser durchlassende Filterzylinder."
V signifies "viel mehr Wasser als normale durchlassende. . . . ."

In response to further inquiries the manufacturers write:

"As to the size of the pores of our filters V, N, and W, we derive our data from the rate of flow of pure filtered water under a certain constant pressure. The figures thus arrived at correspond well with the Poiseuille-law. The question, however, how much the flow of a filter is dependent, apart from the interstices, upon the size and number of lacunae is still an open one."

The earlier Berkefeld candles were fitted with a metal headpiece cemented only to the outside of the kieselguhr cylinder. Differences in the coefficients of expansion of metal, filter mass, and cement on repeated sterilization led to the formation of capillary clefts at the joint. After the publication of severe criticisms (43, 182) the method of cementing the threaded outlet tube inside the hollow candle was adopted. Filters so constructed seem to be satisfactory.

Photomicrographs of sections of filters ground to microscopic thinness have been published by several investigators (61, 49, 80), and show a

substance composed of irregular siliceous granules between which are capillary interstices. Irregularly scattered through the filter substance are larger spaces or lacunae varying from a few micra to possibly 100 micra in diameter. These lacunae are most in evidence in the V and least in the W type filters. It is the opinion of most investigators that these lacunae do not completely traverse the filter wall. The pore diameters calculated from rate of water flow are mean values for the lacunae and intergranular interstices taken together.

Mandler Filters.—The greater rapidity of filtration through kieselguhr filters, as compared with porcelain ones, has been a great advantage where output is of cardinal importance, *e. g.*, in serum laboratories. When the supply of Berkefeld filters was stopped by the war, the Mandler diatomaceous earth filter was developed in America. Here again difficulty was met with in obtaining germproof joints between the metal headpiece and filter. This difficulty has since been overcome, however.

The Mandler filter compound is composed of from 60 to 80 per cent ground kieselguhr, 10 to 30 per cent of asbestos, and 10 to 15 per cent of plaster of Paris; the proportions are varied according to the porosity desired. The ingredients in the form of a fine powder are mixed with a small amount of water. The mixture is then subjected to high pressure, after which it is baked in a kiln at a temperature ranging from 1800° to 3000°F. The candles are subsequently treated with water to wash out the lime (141).

The maker of Mandler filters describes their testing as follows:

"In making our test, we submerge the candle in a glass case that is filled with water. The connection is made from the nipple of the candle to a pipe through which is passed the air from a compression tank controlled by a gauge. It is only this air that is passed to the inside of the candle. The gauge tells us at what pressure air bubbles appear on the outside of the candle in the water. In this way, we test each candle and mark the pounds of pressure on the bottom with a die" (2).

The Mandler filters are made in three grades, "preliminary," air passing through at pressure of 2 to 5 lbs.; "regular" at 6 to 9 lbs.; and "fine" at 10 to 16 lbs. per sq. in. The preliminary candles are not offered as bacteria-tight; they are intended to remove, as far as possible, the coarser material in suspension.

Asbestos Filters.—The Seitz (EK)[2] filter discs have been used in filter presses for some years in the wine industry in Europe. More recently these filter presses have been adopted for the sterilization of large

[2] "Entkeimende."

amounts of serum. Finally smaller filters with Seitz discs have been made for use in experimental work. A detailed description of the construction and operation of the filters is given by Tron (165), and in brief is as follows:

> The Seitz filters consist of thin filtering sheets of asbestos contained in a tinned brass apparatus suitable for holding the unfiltered liquid, for sterile delivery of the filtrate, and for operation at positive or negative pressure. The filtering disc is clamped tightly between two smooth metal rims by screw clamps. Satisfactory use of the Seitz filters has been reported by several workers (167, 157, 165, 63).

A special type of Seitz filter, the Manteufel (104) model, has recently been advertised. In this model two threaded metal parts screw together compressing the asbestos disc between them. Such an apparatus tested by the writer yielded only contaminated filtrates. The Uhlenhuth type, on the other hand, yielded sterile filtrates. Tests with dyes revealed why the Uhlenhuth was better than the Manteufel type. The margins of the asbestos discs in the Uhlenhuth model were deeply depressed by the metal rims which could be pressed together powerfully by the screw clamps; no leakage of dye across this compressed margin occurred. The margins of the filtering discs used in the Manteufel model were less well compressed and permitted leakage of dye. Conceivably the particular Manteufel apparatus tested may have had some special defect; such was not apparent on careful scrutiny, however. Another Manteufel model tested with dye behaved in a manner similar to that of the first. This model seems to be ill-conceived mechanically; the metal rims screw together opposed by friction against the asbestos disc they are compressing; therefore, failure to make a bacteria-tight joint under these conditions is not surprising.

Plaster of Paris Filters.—Kramer's filters (90) with basoid adsorptive properties are made as follows (91):

> A rubber stopper with a single perforation is placed in one end of a glass cylinder. Through the hole in the stopper, a glass tube containing a close fitting glass rod is thrust. A paste is made in the proportion of 75 gms. gypsum ($CaSO_4$ + 2.5 per cent $CaCO_3$) to 25 gms. MgO calcined at 1300°C., in 75 gms. water. The paste is then poured into the cylinder so as to cover the stoppered end and allowed to harden. The glass rod is finally removed from the glass tube, which serves to deliver the filtrate.

An elaborate study of the relative efficiency of various bacterial filters was made as early as 1898 by Woodhead and Wood (181). These workers pronounced the Chamberland, Berkefeld, Garros,

Pukall, and Slack and Brownlow filters effective in preventing the direct passage of bacteria. All, however, permitted growth of non-pathogenic water organisms through them after intervals varying from 2 to 8 days. More recently, 1925, Bedson and Maitland (26), of the British Foot-and-Mouth Disease Committee, write as follows concerning the relative merits of filters:

"For the purposes of this investigation we have been concerned principally with obtaining a sterile filtrate with as high a virus content as possible, and in order to achieve this desideratum we have experimented with different makes of filter candles. Chamberland L 1A, L 2 and L 3, Berkefeld and Mandler (6 to 12 lbs.) candles have been used, and although all three makes have their points, the last named has been found the most generally useful. The Chamberland candles are excellent when new, but soon become blocked and are difficult to clean. The Berkefeld candles have the drawback that they are extremely fragile, though they can be satisfactorily cleaned and used time after time without much appreciable loss in speed of filtration. The Mandler filter, in addition to possessing these qualities, is much more durable than the Berkefeld."

The Foot-and-Mouth Disease Research Committee further report (63):

"Some advantages of using Seitz filters are that they are easy to handle and the trouble of cleaning is dispensed with as the asbestos discs are thrown away after use. The more important point, however, is that they have always been found capable of yielding sterile filtrates when not more than about 20 cc. of slightly contaminated fluid is submitted to filtration. With certain precautions they are efficient for arresting the bacteria of fowl cholera in 10 cc. broth culture. They have several times failed, however, to arrest all the staphylococci in a broth culture when the volume of fluid passed through a single disc amounted to 100 cc."

Ultrafilters.—The use of jelly filters to separate colloids from their solvents and from dissolved crystalloids, and to separate one colloid from another having different sized particles, is known as ultrafiltration. Silicic acid gels and gelatin have been used in some instances. The ultrafilters generally used, however, are made of collodion. The collodion is as a rule deposited in a porous supporting structure, *e. g.*, filter paper (17, 22, 24), unglazed porcelain (25), or alundum (39). The "membrane filters" of Zsigmondy and Bachmann (187) and Zsigmondy (186) are of pure nitrocellulose. Membranes of graded porosity may be obtained both in the Bechhold and the Zsigmondy types (106). Suitable apparatus for conducting filtration through these different kinds of membranes at reduced or at moderate or high positive pressures have been developed. Detailed descriptions of apparatus and methods are given in the references cited.

Electroultrafilters.—Morse and Pierce (117) introduced the method now known as "electrodialysis" for purifying colloids. They passed a high tension direct current through gelatin enclosed in a parchment membrane immersed in pure water. Electrolytes migrated to the electrodes and were washed away by running water. Disturbances in H-ion concentration within the dialyzing vessel occasioned serious difficulty with this method, however. Apparatus designed to overcome this and other objections to electrodialysis by combining it with ultrafiltration have been described by Bechhold (24) and by Bronfenbrenner (39). Electroultrafiltration is more expeditious for removing electrolytes from colloidal preparations than either dialysis or ultrafiltration alone.

The Loss of Material by Filtration—Adsorption[3]

Filtration usually involves some loss of active material. There are many indications that the loss of activity of enzymes, toxins, complement, antibodies, and, in some instances, viruses in passage through the ordinary bacterial filters is due less to mechanical straining out of large particles than to adsorption of the active agents on the walls of the filter pores. A discussion of the mechanism of this adsorption and of the means of minimizing the incident losses necessitates a consideration of the physical-chemical properties of filters.

Composition of Filters.—Kieselguhr, the chief component of Berkefeld and Mandler filters, consists of the siliceous shells of diatoms. It seems probable that the organic material used in the Berkefeld filter mass is burned to ash in the baking of the filter. How much of the plaster of Paris in the Mandler filters remains after the burning and subsequent washing is uncertain. Clay, the basic material of unglazed porcelain filters, "consists essentially of hydrous aluminum silicate with various impurities" (64). Asbestos, described as the chief component of Seitz filter discs, comprises "various silicate minerals of finely fibrous texture and flexibility" (163). The bacterial filters in common use are thus made essentially of metal silicates. Collodion, the usual filtering medium in ultrafilters, is nitrocellulose. Its adsorptive properties have been discussed by Bechhold (18, 22), Hitchcock (77), Grollman (69), Michaelis (110), and Risse (145).

Charge of Filters.—The electrochemical properties of bacterial filters, *i.e.*, their electric charge and their ability to adsorb electropositive and

[3] The interpretation of the mechanism of adsorption in bacterial filters given in this chapter is here developed for the first time, so far as the author is aware. Professor Michaelis has kindly read and approved the manuscript.

electronegative dyes, are those of silicates. The charge of various filters (Berkefeld V, N, and W; Mandler preliminary and regular; Chamberland B and F; Allen; Coors; Maassen; Seitz) has been determined by the writer directly by electroendosmosis in the following manner:

The filters were immersed in N/100 hydrochloric acid, in acetate buffers of pH approximately 3.6 and 5.1, in phosphate mixtures of pH approximately 6.6 and 8.0, and in N/100 NaOH. The movement of liquid through the filter while traversed by an electric current was determined by suitable apparatus (121).

In all cases the water streamed through the filter toward the cathode, indicating that the solution was positively and the filter pore walls negatively charged. The charge was much reduced, however, in the presence of N/100 HCl. This behavior is characteristic of silicates, although in no sense specific. Its explanation in modern terms is that the metal cations of the filter substance have a greater tendency to go into solution in the water than do the silicate anions and in the electric current they are attracted toward the cathode whither they carry water of hydration and some water that is borne along by friction.

Adsorption of Dyes.—It was shown independently by Kramer in 1916 (90) and by Mudd (118), and subsequently by Olitsky and Boëz (137), that bacterial filters will adsorb limited amounts of basic dyes but will allow acid dyes to pass essentially unaltered. If sufficient basic dye is filtered to saturate the adsorbing surface of the filter, however, the dye subsequently passes without hindrance into the filtrate. The reaction is reversible, inasmuch as distilled water passed through such a dye-saturated filter carries dye into the filtrate. The following protocols are illustrative.

A new Berkefeld W candle, 13 cm. × 2.5 cm., completely decolorized 400 cc. of distilled water containing 100 mgms. per liter of dry safranine. The filtrate thereafter deepened in color until it was like the unfiltered solution. Amount of dye adsorbed before first color appeared in filtrate = 40 mgms.

A similar new filter completely decolorized 125 cc. of Ringer-Locke solution containing 50 mgms. of dry safranine per liter. Amount of dye adsorbed before first color appeared in filtrate = 6.25 mgms.

A similar new filter completely decolorized 3000 cc. of methylene blue solution in distilled water containing a total of 20 mgms. of dry dye. The filtrate then deepened in color until it was like the unfiltered solution.

A similar new filter completely decolorized 1525 cc. of methylene blue solution in Ringer-Locke solution containing a total of about 15 mgms. of dry dye.

Filtration in each case was conducted by means of gravity alone. The smaller amount of dye adsorbed from salt solution as compared with the amount adsorbed

from distilled water is to be noted. This is the usual, but not the invariable, effect of the presence of salts on the adsorption of other substances (111, p. 238).

The filter saturated with the distilled water solution of methylene blue was washed for several weeks by allowing distilled water to filter through under gravity. The filtrate had a blue tint throughout this time.

A Seitz disc, 60 mm. in diameter, completely decolorized 410 cc. of a solution containing 10 mgms. of methylene blue per liter of distilled water. Filtrate thereafter deepened in color and when 820 cc. had been filtered it was like the unfiltered solution.

A Seitz disc, 60 mm. in diameter, completely decolorized 230 cc. of a solution containing 10 mgms. of methylene blue per liter of 0.85 per cent NaCl solution. After 480 cc. had been filtered the filtrate was like unfiltered solution.

Seitz discs did not completely decolorize solutions containing 10 mgms. of eosin per liter of distilled water or salt solution. Only the first few cubic centimeters of filtrate were somewhat fainter in color.

The mechanism of the adsorption of dyes on filters seems clear. The basic dyes (methylene blue, safranine, etc.) are salts of organic color cations with inorganic anions such as Cl^-, $SO_4^=$, etc. The acid dyes (eosin, picric acid, etc.) are salts of organic color anions with inorganic cations such as Na^+, K^+, NH_4^+. The silicates have readily replaceable cations as has already been indicated. When, therefore, basic dyes are brought into contact with silicates (114) the strongly adsorbable color cations displace the cations of the adsorbent to form an insoluble dye-silicate.

The reaction between silicates and dyes has been explained by Michaelis (111) in the following manner:

"Ionogenic adsorbents, which may be conceived of as insoluble 'salts,' are represented by such substances as kaolin (silicates of alkaline earths) and the so-called ferric oxide (basic ferric chloride). Such adsorbents behave entirely according to the well known laws of chemical reactions and react with the electrolytes in aqueous solution by means of an exchange of ions. A solution of methylene blue chloride reacts with kaolin according to the following scheme: Alkaline earth silicate + methylene blue chloride = Methylene blue silicate + alkaline earth chloride.

"Since methylene blue silicate is insoluble, and the alkaline earth chloride is soluble, the kaolin exchanges its alkaline earth cation (especially the ever present Ca^{++}) for the methylene blue cation, while the Cl-ion of the methylene blue remains in solution, not as HCl, however, but as $CaCl_2$. An exchange of ions between kaolin and NaCl cannot be demonstrated by analytical methods, although doubtless according to general chemical laws such an exchange can take place. Not a trace of eosin or picric acid is adsorbed by kaolin, for an exchange of ions in these instances would not result in the formation of insoluble salts."

Conversely when NH_4-eosin is brought into contact with basic ferric chloride, the eosin anion is adsorbed to form an insoluble compound and the NH_4^+ and Cl^- go into solution (114).

Adsorbents of the type of the silicates, which are negatively charged in aqueous solutions and which have readily replaceable cations, are called *acidoids* by Michaelis (111). Kieselguhr, porcelain, and asbestos filters belong in this class. Adsorbents like ferric oxide (basic ferric chloride) which are positively charged against aqueous solutions and have readily replaceable anions are called *basoids* by Michaelis. Kramer's plaster of Paris filters belong in this group. Collodion is not regarded as ionogenic itself. Its negative charge is considered to be due to the adsorption of negative ions (110).

To recapitulate, then: the kieselguhr, porcelain, and asbestos bacterial filters owe their negative charge and their ability to adsorb basic dyes to the fact that they are made essentially of salts of soluble metal cations with insoluble silicate anions; the electronegative silicate ions form salt-like compounds with the dye cations.

Adsorption and Filtration of Proteins and Biologically Active Substances.—Protein solutions contain undissociated protein and salts of protein ions with inorganic ions (103, 47). At the isoelectric point of the protein, dissociation is at a minimum and the protein anions and cations are equal in number (108, 111). As the reaction of the solution departs progressively from the isoelectric point of the protein, the percentage of dissociated protein increases. At reactions more acid than the isoelectric point, the dissociated protein is chiefly in the form of multivalent cations capable of forming salts with anions. At reactions more alkaline than the isoelectric point, the protein dissociates chiefly as multivalent anions capable of forming salts with cations.

There is every reason to believe that these relations are of importance in affecting the adsorption of proteins in filters. One may confidently predict that in acid fluids the protein cations will tend to form salt-like combinations with the silicate ions of the filter walls and that these primary valence forces will not be without influence whether the ionized protein is in molecular dispersion, is in micellae, or forms part of the surface of a living agent. Correspondingly, enzymes (81–83), toxins (11, 41), and even viruses (136, 137) have been reported to pass more readily through filters in weakly alkaline solutions than in acid ones.[4]

The results of Davis' (50) studies concerning the adsorption of serum on glass is in accord with the observations reported above. This worker

[4] This statement is not meant to carry any implication as to the chemical nature of enzymes, toxins, and antibodies other than that, so far as present evidence goes, they either are amphoteric or are commonly attached to substances which exhibit the amphoteric properties of proteins.

showed that in strongly acid solutions serum in a dilution of 1:10,000 was as effective in altering the charge of electronegative glass particles by coating them with protein as it was in dilutions of 1:10 or 1:2. If the solutions were near or on the alkaline side of the isoelectric points of the serum proteins, the glass particles were altered only slightly in low serum concentrations, but to a greater extent in higher serum concentrations. His work illustrates (1) that the charge, or dissociation state, of the protein is of importance in the manner above indicated in the adsorption of protein by silicates, and (2) that the charge is not the sole factor in determining the adsorption of proteins by silicates.

The mechanism of the adsorption of electronegative substances by electronegative filter pore walls or by other silicates cannot be adequately explained at the present time. It is relevant, however, to restate the familiar Gibbs-Thomson principle which affirms in effect that any substance which decreases the free energy of a surface will tend to accumulate at that surface.[5] Protein particles are well known for their tendency to condense at boundary surfaces. A familiar example is their stabilizing action on emulsions such as milk; accumulation of protein at such interfaces occurs even though the protein and the oil droplets are both electronegative to the watery phase. Similarly it has been shown (124–126) that electronegative bacteria, the acid-fast ones excepted, are strongly adsorbed in oil-water interfaces and that their behavior in such boundary surfaces is explainable in terms of the interfacial tension relations. Moreover, Eisenberg (58) and Michaelis (109) have shown that bacteria are adsorbed not only by basoid adsorbents but also by kaolin and kieselguhr, and that Gram-positive organisms are much better adsorbed than are Gram-negative ones.

The adsorption of the euglobulin, pseudoglobulin, and albumin fractions of horse serum by various adsorbents has been studied by Landsteiner and Uhlirz (99) who found that with increasing concentrations of each of the proteins, increasing amounts, but decreasing percentages, of the proteins were adsorbed by kaolin. Moreover, the various silicates and other adsorbents showed different adsorptive power for the serum fractions, and the adsorptive capacity of kaolin and talc for euglobulin

[5] This type of adsorption was formerly known as "mechanical" or "physical" adsorption and was distinguished with emphasis from the "chemical" adsorption discussed above. The importance of this distinction is disappearing in the light of modern work which tends to refer both types of adsorption ultimately to "the electromagnetic force-field pattern" or the distribution of attractive and repulsive forces, chiefly electrical, about the atoms and atomic groupings within the molecules of adsorbed substance and adsorbent.

was decreased by being heated to incandescence. Landsteiner and Stanković (98) found that the adsorption by kaolin of dilute horse serum, beef serum, and abrin solutions was enhanced by heating the solutions for a day at about 60°C. The effect of H-ion concentration unfortunately was not noted. It was shown by Rosenthal (146) that egg albumen was strongly adsorbed in Berkefeld and in Reichel filters, while Witte's peptone passed Berkefeld filters without loss. The adsorption of egg white both by kaolin and by iron hydroxide was shown by Biltz (32) to be incompletely reversible. Michaelis and Rona (113) reported that the adsorption of albumoses by charcoal, mastic, iron hydroxide, and kaolin was almost irreversible. Hemoglobin has been reported as traversing Chamberland candles more readily on the alkaline than on the acid side of its isoelectric point (137).

Precise studies of the adsorption of proteins in the pores of collodion membranes have recently been made by Hitchcock (77–79) with the following results: The amount of protein adsorbed was maximal at its isoelectric point. The amount adsorbed decreased steadily with increasing alkalinity, but with increasing acidity it decreased to a minimum, after which it again increased in strongly acid solutions. The amount of protein adsorbed from weakly acid solutions was increased by the presence of neutral salts. The increase in amount of protein adsorbed with increasing concentration did not fit the Freundlich adsorption isotherm, but did fit Langmuir's adsorption formula. The permeability for water of protein-coated collodion membranes was maximal at the isoelectric point of the protein; it was lessened by acids or by alkalis that caused swelling of the protein in the collodion pores. These studies of Hitchcock indicate the manner in which bacterial filters should be studied.

Enzymes.—Michaelis and Ehrenreich (112) studied the adsorption of various preparations of enzymes by kaolin, an electronegative adsorbent, and by aluminum hydroxide, an electropositive adsorbent. Malt diastase was not adsorbed by kaolin in neutral and alkaline solutions. It was completely adsorbed, however, in acid solutions. Invertin and pepsin were adsorbed by aluminum hydroxide but not by kaolin, thus evidencing an acid nature. Trypsin and salivary diastase were adsorbed by both adsorbents. The charge of the enzyme preparations, determined by cataphoresis, was in harmony with the results of the adsorption experiments. Willstätter (173) has pointed out, however, that the above results apply only to impure enzymes and that the adsorption behavior of carefully purified enzymes may be different from

that of impure ones. In regard to the purified ones, he says: "Different enzymes exhibit at the same time acid and basic properties with fine gradations. On this their separation by adsorption may be based."

The early literature concerning the filtration of enzymes is given by Levy (100). More recently, Holderer (81) studied the filtration of impure enzyme preparations at different reactions. Sucrase from *Aspergillus niger* in a medium neutral to phenolphthalein (pH = approx. 8.3) was found to pass a Chamberland F bougie unaltered; in a medium neutral to methyl orange (pH = approx. 4.4), however, it was almost completely retained in the filter. Work with other carbohydrate-splitting enzymes confirmed these observations (82). The greater filterability of the enzymes in weakly alkaline media was at first attributed to assumed changes in the state of dispersion due to altered reaction (81). Later, however, Holderer (83) found that enzyme preparations to which 10 per cent egg white or 1 per cent peptone was added could be filtered through a Chamberland F bougie without diminishing its potency even at an acid reaction at which the enzyme preparation alone was reduced by filtration to 4 per cent of its original activity. Moreover, it was found that preliminary passage through the filter of 15 cc. of acid solution of albumen (prepared by diluting egg white with 3 volumes of water, filtering it through a metal cloth and then through paper, and making it acid to methyl orange with HCl) prepared the filter in such a manner that sucrase and emulsine were allowed to pass even at acid reactions. These observations were justly considered by Holderer to be evidence of the fact that the loss of activity of an enzyme upon passing through a filter is dependent largely on adsorption phenomena.

Toxins and Antitoxins.—The adsorption of tetanus and diphtheria toxin, antitoxins, and toxin-antitoxin mixtures by various natural and prepared silicates has been studied by Zunz (188, 189). According to this observer, kaolin, kieselguhr, talc, and clay adsorb neither diphtheria toxin nor its antitoxin. Kaolin and clay adsorb both tetanus toxin and antitoxin. Talc adsorbs only tetanus toxin, kieselguhr only tetanus antitoxin. Tetanus lysin was only slightly, and tetanus antilysin and tetanus toxin-antitoxin mixtures were not detectably adsorbed by any of these silicates. Since the hydrogen ion concentration was not controlled the results of these experiments lose much of their significance. Aubel and Colin (11) reported that diphtheria toxin and dysentery endotoxin passed a filter in alkaline but not in acid solutions. Bronfenbrenner (41) found botulinus toxin to be filterable at pH = 7 to pH

= 5.5 but not at pH = 4. Filterability of the toxin could not be tested in alkaline solutions because it rapidly becomes inactive above pH = 8.

Antibodies and Complement.—Many reports concerning the adsorption of antibodies, other than antitoxins, and of complement have appeared in the literature. Failure, however, to control certain experimental conditions, *e. g.*, H-ion concentration, detracts from their value. Nevertheless, some of the reports will be reviewed briefly. Biltz, Much, and Siebert (33) found that antibodies were best adsorbed by metal sols. Andrejew (3, 4) showed (1) that a variety of antibodies was partially adsorbed by kaolin or kieselguhr, (2) that a greater percentage of antibody was adsorbed from dilute serum than from concentrated serum, and (3) that complement was more easily adsorbed than antibodies. Muir and Browning (128) found (1) that in the early stages of filtration complement was almost entirely retained in the filter and could not be recovered, (2) that after filtration had progressed for a period of time the complement appeared in the filtrate in undiminished concentration, (3) that serum, diluted with an equal volume of 10 per cent NaCl solution, upon filtration lost practically no complement, and (4) that complement was held in the filter while hemolytic amboceptor passed through readily. Arloing and Langeron (9) confirmed the observations of Muir and Browning in regard to filtration of complement and hemolytic amboceptor and concluded that the particles of complement are larger than those of antibodies. Such a conclusion, however, seems to be unwarranted.

Filterable Viruses.—A lengthy discussion of the phenomena observed in the process of filtering proteins, enzymes, toxins, and antibodies may seem out of place in a book on filterable viruses. On the other hand, inasmuch as no filterable virus has been obtained in a pure state, and since all of them are usually studied in emulsions containing large amounts of protein and other substances, such a discussion becomes absolutely necessary. Workers, therefore, must keep in mind the fact that viruses may be adsorbed on other particles and that phenomena observed in filtration experiments may be due not to the action of the viruses but to that of the particles on which they are adsorbed.

A number of experiments have been described dealing with the adsorption of viruses. For instance, Lewis and Andervont (101) reported that the viruses of vaccinia and fowl-pox are adsorbed by powdered Berkefeld filters, kaolin, and charcoal, while the virus of Rous' sarcoma is adsorbed by aluminum hydrate but not by the silicates. Such differences in adsorption as those just noted may account for

differences in the filterability of certain viruses. It seems unnecessary to review all the reports concerning the adsorption of viruses as no final conclusions can be drawn in regard to the matter at the present time. Some idea, however, concerning the amount and nature of the work that has been done in this field can be obtained from the chapters on bacteriophage and foot-and-mouth disease.

Used Filters.—It is to be noted that the foregoing discussion dealt with the adsorptive properties of bacterial filters themselves and is thus applicable chiefly to filtrations of relatively short duration through clean filters. The continued filtration of liquids containing adsorbable materials will of course tend to saturate the adsorbing surface, and liquids containing bacteria or cellular detritus will eventually form a layer of deposit on the outer surface of the candle. The straining-out and adsorptive properties will then depend largely on the deposited material. It is a familiar saying among workers in industrial filtration that filtration is performed not by the filter but by the deposit or "cake." It has been shown (Hixson, A. W., Work, L. T., and Odell, I. H., Mechanism of filtration, *Tr. Am. Inst. Mining Engineers*, 1925–26, lxxiii, 225) that particles may form a "cake" over filter pores many times the diameter of the particles; the tendency to "cake" increases with the percentage of solids in the filtering fluid. For discussion of the principles of technical filtration see Hatschek (71, 72).

Filterability As an Indication of Size

Discussion of filtration has thus far been concerned with the retention by molecular forces of matter too finely subdivided to be mechanically strained out by the filter pores. Nevertheless it is obvious that there must be a range within which the size of filtering particle and the size of filter pores are so related that sieve action assumes increasing importance in filtration. With increasing size of the particles a limit must eventually be reached beyond which no rigid particle can pass the filter. Under conditions of routine use of filters, recorded work in this field seems to indicate that the critical range of particle size within which sieve-like action assumes increasing and finally exclusive importance lies somewhere in the realm bounded on the one hand by the size of the smaller filterable viruses and on the other by that of ordinary bacteria. Within this range also, the limits of microscopic visibility fall (150, 13).

The better bacterial filters were early shown to hold back ordinary organisms when the filtration was conducted under moderate pressure

for a short period of time. A class of disease incitants, however, through the observations of Iwanowski (86) and Beijerinck (27) was found to be ultramicroscopic and to traverse filters with great ease. The conception of a small filterable stage in the life cycle of a large microörganism was presented by Schaudinn (151) on the basis of studies of protozoa (*cf.* 134). The misconception developing from these observations gained and still has currency, that the lack of sterility of filtrates from proper bacterial filters *ipso facto* justifies the assumption that the filter-passing organism is either an ultramicroscopic virus or an invisible form in the life cycle of a visible organism (42).

In discussing the filterability of microörganisms[6] an important distinction is to be made between the forms which may traverse the more porous bacterial filters in numbers sufficient only to inoculate the filtrate and those which reach the filtrate in almost undiminished concentration. The capability of inoculating the filtrate under favorable conditions is possessed by many microörganisms and does not of itself imply invisibility or necessitate the assumption of a mysterious "ultramicroscopic stage." Passage of infectious agents into the filtrate, in full or nearly full strength, is a different matter, however, and does imply properties other than those of even very small visible microbes. Failure to keep these facts in mind has led to confusion in the literature.

Visible Organisms Capable of Seeding the Filtrate.—Many organisms are able to penetrate bacterial filters if contact between them is sufficiently long continued, especially if nutrient material suitable for the growth of the organism is present in the filter pores (95, 159, 96, 45, 61, 80, 44, 161). Under these conditions the filtrate is at first sterile; portions collected on the second or third day may show a few organisms; portions taken on subsequent days show increasing numbers of bacteria; and the final portions of filtrate, because of multiplication of bacteria in the filter pores, may be more heavily contaminated than the unfiltered material. The growth of bacteria through membranes, the size of whose pores was estimated by the air-bubbling method, has been studied by Heymans (76).[7] This worker reported that bacteria traversed membranes whose pore diameters were estimated to be 0.1 μ, and, to explain this singular result, advanced his theory of "ultradiapédèse

[6] The term "microörganism" may be understood in this chapter to include viruses; no implication as to the nature of viruses is intended.

[7] He unfortunately used Bechhold's (18) formula for pore diameter in its original incorrect form (discussed later in the chapter) which gives values ten times too small.

microbienne," endowing bacteria with the power of ameboid migration. Filtration experiments extending over a great length of time should, therefore, be conducted at low temperatures (181).

In addition to the narrowness of the filter pores, the length and tortuosity of the channels through the filter walls are also factors in the retention of bacteria by filters. Thus Rosenthal (146), using special filtering plates of different thicknesses made of Berkefeld, Chamberland, and German porcelain masses, found that the ability of these plates to retain *Sp. parvum* increased with their thickness. Similarly Novy and Knapp (135) obtained infective filtrates of *Sp. obermeieri* in all cases in which they used Berkefeld filters sandpapered until the walls were very thin, but in only some instances when stock candles were employed.

Various conditions favor the passage of bacteria through the filter pores and may bring about inoculation of the filtrate, *e. g.*, the small size of the microörganisms, as in the case of *Bact. pneumosintes*, 0.15 to 0.3 μ in length, a half to a third as large in diameter (138, 139), and other minute anaërobes isolated by Olitsky and Gates (140), Holman and Krock (84), and others (116). Adsorption is also a factor even in the filtration of bacteria (20, 21, 118, 85). The latter workers found that Mandler filters whose pores contained a trace of oil were pervious to *B. prodigiosus* under circumstances in which clean filters retained this organism. Furthermore, motility, in addition to small size, may increase the ability of certain organisms to pass through filters, as in the case of *Sp. parvum* of v. Esmarch (61), diameter 0.1 to 0.3 μ, and the filterable spiral organisms and the protozoa of tap water discovered by Borrel (37). Further evidence of the relation of motility of an organism to its filterability is shown by the behavior of *V. percolans* (127). This vibrio resembles in appearance and motility the vibrio of Asiatic cholera. It is smaller, however, and includes some coccoid individuals of 0.3 μ diameter or less. It passes regularly through V, but not through N or W Berkefeld filters. When its motility was suppressed by acid, anesthetics, or cold, sterile filtrates were obtained under circumstances that yielded inoculated filtrates in the controls (119). Thus, when a culture is drawn through an irregular capillary bed under conditions in which a few organisms may or may not pass through, motility may be a critical factor in determining passage. Motility doubtless aids the passage by combating the tendency of the organisms to adhere to the pore walls, or to wedge themselves across the pores.

Slenderness, motility, and flexibility are characteristics that probably enable certain organisms to pass filters, *e. g.*, *Sp. obermeieri* (135), *Sp.*

elusa (179), *Sp. biflexa* (180), *Leptospira icterohaemorrhagiae* (130, 172), and Noguchi's (131) *Leptospira icteroides*. Wolbach (178) kindly permits the inclusion here of an account of some unpublished work with spirochetes:

"Successive filtrations of *Spirocheta elusa*, that is, filtering the culture that developed from the preceding filtrate, extended from January 11, 1915 to March 3, 1917, and during that period 97 filtration experiments were made. I had no quantitative method at my command to apply to it, and of course there are many variable factors in cultures, but the percentage of positive filtrations increased in a very striking way so that at the end of that period the results were uniformly positive. With Berkefeld filters, 20 lb. pressure, time, 1 minute, the spirochaetes came through as such. As a matter of fact, one could demonstrate spirochaetes in the filtrate, with a dark field, in the second cubic centimeter of filtrate, using a pressure of only 2 feet of water. That experiment I did repeatedly. In every experiment *Bacillus prodigiosus* was used as control. Furthermore I have demonstrated the spirochaetes in pores throughout the thickness of the Berkefeld filter. I believe that the filterability of spirochaetes is due to flexibility and mobility, i. e., kinetics versus the forces which hold bacteria to the sides of the pores."

Finally, slenderness, motility, and flexibility of the organisms may not suffice and high filtration pressure may be necessary for inoculation of the filtrate. Todd and Wolbach (164) came to the conclusion that: "*Sp. duttoni* in an infective form can be forced through a Berkefeld filter by pressures of over 50 pounds to the square inch. *Sp. duttoni* cannot be filtered through a Berkefeld filter in an infective form by atmospheric pressure."

Pleomorphism and Filterability.—References to "filterable phases" of visible microörganisms are found frequently in the literature; well-authenticated instances are rare, however. As has been pointed out above, the infectiousness of a filtrate or its capacity to show growth on incubation does not necessarily require as an explanation the assumption of "filterable phases" in the life cycle of the organism under study.

In the year of publication of Schaudinn's prediction that certain minute stages of trypanosomes would prove to be filterable, Novy and MacNeal (133) reported that in cultures of *Tr. lewisi* minute, actively motile forms, 2 to 5 μ in length, were observed and that 3 out of 9 Berkefeld filtration experiments yielded infective filtrates. Similar experiments with cultures of *Tr. brucei* and with suspensions of the organs of animals infected with *Tr. brucei* gave negative results. More recently, however, in 20 of 72 filtration experiments with organ macerates of guinea pigs dead of *Tr. brucei*, Reich (144) obtained infective filtrates. On the other hand, 7 filtrates from highly infective blood were negative.

The results of Reich's work indicate that the source in the animal from which the material to be filtered is obtained may determine the filterability of the infectious agent.

A report of pleomorphism, well documented with data concerning cultivation and filtration, has recently appeared in Lewis' (102) account of work with a protophyton. He describes a "pleomorphic microorganism certain forms of which are within the range of ordinary microscopic visibility, other forms at the limits of visibility, would, taken alone, be essentially invisible. Under certain conditions forms capable of regenerating the complete culture are filterable through Berkefeld N filters."

Amazing claims for pleomorphism of the tubercle bacillus have been made by Vaudremer and by Bezançon and Hauduroy (30). Vaudremer reported that tubercle bacilli inoculated into the depth of tubes of a decoction of potato (168) produce an abundant growth of non-acid-fast "mycelial forms" without tuberculin formation (169). Filtrates obtained by passage of the cultures through Chamberland L 3 candles when inoculated into new potato water reproduce the "growth" (170, 74, 30). Attempts by the writer (1927) to confirm this work have shown that the described course of events (both macroscopic and microscopic) is in truth uniformly produced in the potato water, but that it is produced also with equal fidelity in uninoculated media. In a series of experiments in which potato water and 2 per cent peptone water were inoculated with various cultures of tubercle bacilli and were, in each case, matched with control tubes which had never been opened after autoclaving, the described deposit regularly appeared in all tubes without significant difference between the inoculated and uninoculated tubes. In potato water from which the precipitate had been allowed to settle out before inoculation with tubercle bacilli, the described course of events did not occur. The tubercle bacilli used as inocula were shown to be viable by seeding them on Dorsett's and on Petroff's media. The "mycelial forms" of the tubercle bacillus appear, then, to be sterile precipitates.

The infectivity for guinea pigs of tuberculous material passed through Berkefeld or Chamberland L 2 and L 3 filters has been repeatedly reported. A review of the work in this field has been published by Hauduroy (73). Final conclusions, however, in regard to filterable forms of tubercle bacilli cannot be given at the present time.

Ultramicroscopic Viruses.—It has been reported that certain viruses pass through kieselguhr or the more porous porcelain filters in almost

undiminished concentration, *e. g.*, the virus of foot-and-mouth disease (137), of tobacco mosaic (54, 55), and the bacteriophage (10). The virus of foot-and-mouth disease has been reported to pass through even the less porous porcelain filters, Chamberland L 9 and L 11 (137). Such behavior on the part of viruses leads to a presumption amounting almost to certainty that they are too small for microscopic resolution with visible light.[8] Furthermore, it seems also to indicate that there is very little adsorptive affinity between the filters and these viruses. The converse proposition, namely, that inability to pass the filters without diminution in concentration indicates relatively large or visible dimensions, though often postulated, is of course invalid, inasmuch as the loss may be due merely to adsorption.

In attempting to delimit more narrowly the size of ultramicroscopic viruses, a few investigators have very properly turned to ultrafiltration, *e. g.*, Andriewsky (5), Duggar and Karrer (55), Olitsky and Boëz (137), Bronfenbrenner (40), Zinsser and Tang (183). By passing colloidal suspensions and solutions through ultrafilters of graded porosity, the effects of adsorption being guarded against, Bechhold (17) has shown that colloidal preparations fall into a series[9] which corresponds at least roughly to the size of their particles estimated from their known constitution and from ultramicroscopic data. Although the absolute particle size so estimated is to be taken with reserve (*cf.* Bechhold's criticism of ultramicroscopic estimates of particle size (18)), ultrafiltration is perhaps the best method we have at present for approximating the size of the ultramicroscopic viruses.

[8] Demonstration by ultraviolet photography may still be a possibility, however (14).

[9] Fortunately the estimates of particle size for this series of colloidal suspensions are not based upon the incorrect air-pressure formula discussed below. The series given by Bechhold in 1926 is different from the series of 1907, however, Bechhold (24) warns that, when fluid particles which have a low interfacial tension against the dispersing phase are being ultrafiltered, the size of the ultrafilter pores gives no definite indication of the size of the retained particles. Thus a cloudy lecithin emulsion in which the individual drops were several μ in diameter at a pressure of 150 gms./cm.^{2} passed an ultrafilter tight for hemoglobin. The greater the pressure the more turbid the filtrate. This was explained as due to the droplets assuming a filiform shape on being pressed into the filter capillaries. Zsigmondy has described (185) methods of preparing colloidal gold hydrosols containing uniform particles whose size can be varied at will over a considerable range; he suggests such hydrosols for estimating the pore size of ultrafilter membranes (187). *Cf.*, however, Zsigmondy (185). A method of determining colloidal particle size has been described by Svedberg (162) also.

Attempts to Estimate the Pore Size of Filters.—The height to which water will rise in a capillary tube is proportional to the capillary constant and inversely proportional to the radius of the tube. "Furthermore, if water fills a short length of capillary, it will require the hydrostatic pressure of a column of water equal to the height to which water would ascend in such a capillary, to force it out. By measuring the pressure required to force the water out of a membrane saturated with water we can then estimate the radius or diameter of the pores in that membrane" (31). This relation (Jurin's law) has been proposed by Barus (16), by Bechhold (18), and more recently by Albert Einstein and Mühsam (57) as a basis for estimating the pore size of diaphragms.

The form of the equation which has found most common application, that of Bechhold, unfortunately contains in the denominator a factor 10^5 which should be 10^4. This error was pointed out by Bigelow and Bartell (31) and has been acknowledged by Bechhold (23). The pore diameters calculated from the original equation are thus ten times smaller than those calculated from the correct equation. The correct equation reduces to the form:

$$D = \frac{2.190}{p}$$

in which D is the diameter of the capillary in millimeters at its narrowest point, and p is the pressure, expressed in millimeters of mercury, required to force air through the capillary when filled with pure water at room temperature.[10]

Values for the diameter of glass capillaries calculated by Bigelow and Bartell (31) from the formula above agreed well with those obtained by measurements made with the aid of a microscope. The pore diameters of 9 samples of unglazed porcelain were also estimated by means of the formula and were found to vary from 0.19 to 1.18 μ. In proposing the air-pressure method for the determination of pore size, Einstein and Mühsam state that one assumes that the pore at its narrowest point is circular in cross-section, a condition which is not strictly fulfilled. They calculated that the minimal pore diameter of a porcelain filter ("Tonfilter") is 0.7 μ when the air-pressure method is used, and about 7.0 μ

[10] The formula as it appears in the works of Bechhold (18, 22), Heymans (76), Mudd (119), and Holman and Krock (85) is in each case incorrect in some particular. It is correct in Meyeringh (106), Rosenthal (148) and Bechhold (24). The form given above is a simplification of the correct formula given by Bigelow and Bartell (31).

when determined from the rate of filtration of water (Poiseuille's law).[11] It has been pointed out by the writer that the presence of any substance in the filter pores which lowers the surface tension of water causes the pore diameters, calculated by the air-pressure formula, to appear larger than they really are. Actually it has been found that partially plugged filters may emit bubbles at lower pressures than new ones. With new kieselguhr filters it is improbable that an error is thus introduced, however, since the surface tension of the effluent water from two new Berkefeld candles, determined through the kindness of Dr. du Noüy, proved to be that of pure water.

Calculation of the size of pores in three collodion membranes by Bartell and Carpenter (15), using the air-pressure formula, led to estimates of their diameters as 0.701 μ, 0.934 μ, and 1.681 μ, respectively. Hitchcock (79), using similarly prepared collodion membranes, estimated from data based on Poiseuille's law, that their pore diameters were from 3 to 20 $\mu\mu$ in length. The assumptions involved in each method are discussed by Hitchcock. For collodion membranes, at least, he prefers the method based on Poiseuille's law.

Bechhold in his original paper on the permeability of collodion ultrafilters (18) points out that estimates of the pore diameters of certain filters based on their permeability to collargol (the particle size of which had been estimated ultramicroscopically) were about ten times smaller than calculations based on the air-pressure method. Had he used the air-pressure formula in its correct form, the discrepancy would have been ten times greater or of the same order as the discrepancy between the estimates of Bartell and Carpenter and of Hitchcock. It may be said that the air-pressure method involves the following assumptions whose validity for the narrow-pored collodion membranes is at best extremely doubtful: (a) that the contact angle between water and collodion approximates zero; (b) that the pore width is large in proportion to the range of molecular action; (c) that there is nothing present in the pores to lower the air-water surface tension; (d) that no stretching of the pores

[11] The pore diameter calculated by the air-pressure method (Jurin's law) is the diameter at the narrowest point of the capillary channel through which the bubbling air has passed. Since the pressure at which a small number of streams of air bubbles are emitted by the filter is usually taken as endpoint, the values obtained apply correspondingly to the narrowest points of the largest channels which completely traverse the filter wall. The pore diameter calculated from the rate of flow of water through the filter (Poiseuille's law) is the average diameter of all the spaces (intergranular, gross lacunae, etc.) through which water traverses the filter wall.

occurs. Moreover, the pore diameters estimated by this method have usually been larger than is credible. The pore diameters of Zsigmondy membrane filters calculated from air-pressure data by Meyeringh (106) are an exception to this statement. His figures are quite in accord with what one would expect from the behavior of the membranes in filtering bacteria.

It is somewhat questionable as to how accurate the air-pressure method is in determining the pore size even of siliceous filters. The results of Bigelow and Bartell seem to indicate, however, that the estimates are at least of the right order of magnitude. After the pressures at which streams of bubbles came from a number of bacterial filters had been determined, the writer, assuming the applicability of the formula based upon Jurin's law, calculated the intergranular diameters.

All filters were new. Distilled water was drawn through each filter until it was thoroughly wet. The outlet tube of the filter, still immersed in distilled water, was then connected to an air line. The air pressure was very slowly raised with the aid of the arrangement described by Krock and Holman (94) until bubbling began on the outside of the candle. Pressure was read on an open mercury manometer. The pressure at which six streams of air bubbles came from the outside of the candle was arbitrarily taken as the endpoint. The filter was then wet again by drawing distilled water through it and the determination was repeated. Each value given in Table I is the result of three concordant tests.

The Berkefeld filters frequently emitted bubbles from the collar margin at pressures considerably below the bubbling pressure on the candle proper. This occurred less frequently in the Mandler filters tested. The Berkefeld filters, V, N, and W, fell into definite groups in regard to bubbling pressure which corresponded with their supposed porosity. Such was not the case with a batch of Berkefeld filters tested by the writer in 1923 (119). At that time little or no correspondence existed between designated porosity and bubbling pressure of the filters. When questioned on this point the Berkefeld Gesellschaft (28) wrote: "During the last two years increased attention is being paid to the testing of all our laboratory filters."

The intergranular diameters of the Berkefeld W filters shown in Table I agree well with the average pore diameters estimated by the manufacturers by the method based upon Poiseuille's law and given earlier in the chapter. The intergranular diameters in the table are considerably less than the average pore diameters given by the manufacturers for the N type candles and much less for the V type. The greater number of gross pores or lacunae in the N and V type filters, which enter into

TABLE I

TYPE OF FILTER	SIZE	MANUFACTURER'S DESIGNATION	BUBBLING PRESSURE, (MM. OF Hg)	CALCULATED PORE DIAMETER	AVERAGE PORE DIAMETER*	REMARKS
				μ	μ	
Berkefeld	2½ x ⅝ in.	V	260	8.4		
Berkefeld	2½ x ⅝ in.	V	505	4.3	6.3	Stream at collar margin at 370 mm. Hg
Berkefeld	2½ x ⅝ in.	V	285	7.7		Stream on candle at 270 mm. Hg
Berkefeld	2½ x ⅝ in.	V	330	6.6		Stream on candle at 317 mm. Hg
Berkefeld	2½ x ⅝ in.	N	515	4.3		
Berkefeld	2½ x ⅝ in.	N	515	4.3		Stream at collar margin at 477 mm. Hg
Berkefeld	2½ x ⅝ in.	N	550	4.0	4.0	Stream between collar and end-piece at 401 mm. Hg
Berkefeld	2½ x ⅝ in.	N	600	3.6		Stream between collar and end-piece at 473 mm. Hg
Berkefeld	2½ x ⅝ in.	W	770	2.8		Stream at collar margin at 635 mm. Hg
Berkefeld	2½ x ⅝ in.	W	685	3.2	3.3	Stream at collar margin at 393 mm. Hg
Berkefeld	2½ x ⅝ in.	W	630	3.5		
Berkefeld	2½ x ⅝ in.	W	540	4.1		Stream at collar margin at 50(mm. Hg
Mandler preliminary	5 x 1 in.	4 lbs. (205 mm. Hg)	230	9.5		
Mandler preliminary	5 x 1 in.	4 lbs. (205 mm. Hg)	240	9.1		
Mandler preliminary	5 x 1 in.	5 lbs. (260 mm. Hg)	320	6.8		Stream on candle at 303 mm. Hg

Mandler regular	$2\frac{1}{2}$ x $\frac{5}{8}$ in.	6 lbs. (310 mm. Hg)	320	6.8		
Mandler regular	$2\frac{1}{2}$ x $\frac{5}{8}$ in.	8 lbs. (415 mm. Hg)	405	5.4		
Mandler regular	$2\frac{1}{2}$ x $\frac{5}{8}$ in.	9 lbs. (465 mm. Hg)	485	4.5		Stream at collar margin at 455 mm. Hg
Mandler regular	$1\frac{3}{8}$ x $\frac{5}{8}$ in.	9 lbs. (465 mm. Hg)	520	4.2		
Chamberland	155 x 15 mm.	L 3	895	2.4		
Chamberland	155 x 15 mm.	L 3	815	2.7		
Chamberland	75 x 15 mm.	L 3	850	2.6*	2.7	
Chamberland	75 x 15 mm.	L 3	860	2.5		
Chamberland	55 x 10 mm.	L 3	690	3.2		
Chamberland	205 x 25 mm.	F	1300	1.7		
Chamberland	205 x 25 mm.	B	>1800	<1.2		

* The averages given in the sixth column are calculated from the original data, and are not quite the averages of the rounded values given in the fifth column.

TABLE II

PRESSURE, 0–3 ATMOSPHERES	PRESSURE, 50–100 ATMOSPHERES
B. prodigiosus: length 0.8–1.3μ; breadth 0.3–0.4μ; motile	
Capillary 1.6μ: pos. Capillary 1.3μ: neg. Capillary 1.0μ: neg.	Capillary 1.6μ: pos. Capillary 1.3μ: pos. Capillary 1.0μ: pos. Capillary 0.6μ: pos. Capillary 0.5μ: neg. Capillary 0.3μ: neg.
B. fluorescens: length 1.3–1.8μ; breadth 0.3–0.4μ; motile	
Capillary 1.6μ: pos. Capillary 1.3μ: neg. Capillary 1.0μ: neg.	Capillary 1.6μ: pos. Capillary 1.3μ: pos. Capillary 1.0μ: pos. Capillary 0.6μ: neg. Capillary 0.3μ: neg.
Hay bacillus: length 2.4–5.0μ; breadth 0.4–0.8μ; motile	
Capillary 3.4μ: pos. Capillary 2.4μ: pos. Capillary 2.1μ: neg. Capillary 1.9μ: neg. Capillary 1.6μ: neg.	Capillary 3.4μ: pos. Capillary 2.4μ: pos. Capillary 2.1μ: pos. Capillary 1.9μ: neg. Capillary 1.6μ: neg.
Cocci: diameter 0.6–1.0μ	
Capillary 1.6μ: pos. Capillary 1.3μ: neg. Capillary 1.0μ: neg.	Capillary 1.6μ: pos. Capillary 1.3μ: pos. Capillary 1.0μ: neg. Capillary 0.6μ: neg.
Yeast 740: length 4.0–8.0μ; breadth 3.2–4μ	
Capillary 5.0μ: pos. Capillary 4.6μ: neg. Capillary 4.2μ: neg. Capillary 3.4μ: neg.	Capillary 5.0μ: pos. Capillary 4.6μ: pos. Capillary 4.2μ: neg. Capillary 3.4μ: neg.
Yeast S.: length 4.0–9.6μ; breadth 2.4–3.2μ	
Capillary 5.0μ: pos. Capillary 4.2μ: pos. Capillary 3.8μ: neg. Capillary 3.0μ: neg.	Capillary 4.6μ: pos. Capillary 4.2μ: pos. Capillary 3.8μ: pos. Capillary 3.0μ: neg.

the average pore diameter calculations, no doubt explains these discrepancies. The mean pore diameter of a Berkefeld filter of the less porous type has recently been estimated by v. Angerer (8) by means of Poiseuille's law, and reported to be 1.06 μ.

The figures in parentheses in the third column of Table I show that the bubbling pressures recorded by the manufacturers of the Mandler filters are slightly below the pressures actually observed. This slight difference is intentionally introduced by the makers as a margin of safety.

An extremely careful study of the penetration of bacteria into straight glass capillary tubes of measured internal diameter has been made by Hofstädter (80). His results are summarized in Table II. He found no difference in the minimal tube diameters into which bacteria penetrated without pressure and under a pressure of about 3 atmospheres. Furthermore, the minimal tube diameters were surprisingly in excess of the diameters of the bacteria. Under pressures of 50 to 100 atmospheres smaller tubes were penetrated, but the minimal diameters of these tubes were still well above the diameters of the bacteria. In view of the results of the experiments with straight capillaries, the excess in diameter of the tortuous pores through bacterial filters over the diameters of the bacteria which ordinarily traverse them becomes less surprising.

Technique of Filtration

Testing of Filters.—It is well to force distilled water through new filters to rid them of the pulverized material. If air is forced through the filter while still immersed in water a gross defect will be revealed by the appearance of a coarse stream of bubbles at a comparatively low pressure. This air test can be conducted quantitatively and the diameters of the pores of the filters can be estimated without excessive trouble.

Sterilization.—Dry sterilization of bacterial filters and accessory apparatus is ordinarily impracticable because of the presence of necessary rubber fittings. Prolonged exposure in a steam oven (Arnold sterilizer) may be used. The autoclave is more certain to produce sterilization, and if the temperature is raised and lowered slowly the risk of damage to the filter is not great. A possible exception to this statement is the Berkefeld, whose cemented joints are said to be easily injured in the autoclave (148). The writer must confess that he has autoclaved Berkefeld candles routinely (119) and that such filters

behaved satisfactorily on subsequent test with *B. prodigiosus* and other bacteria.

Control Organisms.—When interest in bacterial filters centered on the problem of their ability to protect against water-borne diseases they were commonly tested by cholera vibrios or organisms of the colon-typhoid group. For insuring the bacterial tightness of filters used in studying viruses, it is desirable to use a small organism of distinctive characteristics which may be simply cultured, handled, and identified. The control organism is usually mixed with the material in which the presence of a filter-passer is suspected. British and American workers frequently use *B. prodigiosus*. When a more severe test of the filters is desired, use may be made of *V. percolans* (127, 166) which passes Berkefeld V candles, or *Sp. parvum*, which is said to pass through all types of Berkefeld candles. For certain purposes it may be desirable to use a series of test organisms of graded filtration properties. Thus Rosenthal (146) used *B. prodigiosus*, *B. pyocyaneus*, the bacillus of swine erysipelas, and *Sp. parvum*. P. Schmidt (154) used staphylococci, bacillus of fowl cholera, *B. phosphorescens*, and *Sp. parvum*.

Preparation of Material for Filtration.—If the material to be filtered is associated with tissue cells thorough grinding, for instance with sand in a mortar, may be required. In order to prevent clogging of the filter by cell detritus or other coarse material, the large particles in the emulsions should be removed by centrifugation or by a preliminary filtration through filter paper, paper pulp, paper pulp and sand (70), a coarse filter candle such as a Mandler preliminary, Berkefeld V, or Chamberland L.

Arrangement of Filters.—For the sterilization of liquids in large quantities, bacterial filters are commonly mounted in reservoirs arranged for the use of reduced or positive pressure. For laboratory filtration on a small scale, the filters, connected by an air-tight line to a suitable filtration vessel, may simply be thrust into the liquid to be filtered. Kieselguhr filters are more commonly mounted in a glass mantel as shown in Fig. 1; the endpiece is thrust through a perforated rubber stopper and the latter inserted in a filtration vessel, usually a conical flask with a side tube for connecting it with the suction apparatus.

The arrangement of the apparatus shown in Fig. 1 has been found superior to the conventional one both in convenience of operation and in accuracy of control over the process of filtration (122). The reduced pressure is shown by the manometer. The filtrate is received into a

graduated vessel. Filtration may be interrupted at any moment by stopping the suction pump and opening the stopcock on the trap to equalize pressure. Measured volumes of the filtrate may then be inoculated sterilely into any desired number of culture tubes through the delivery tube with protective apron. The pinchclamp is released, the stopcock closed, and suction is continued until another volume of filtrate is collected, and so on indefinitely. Since it has been found in

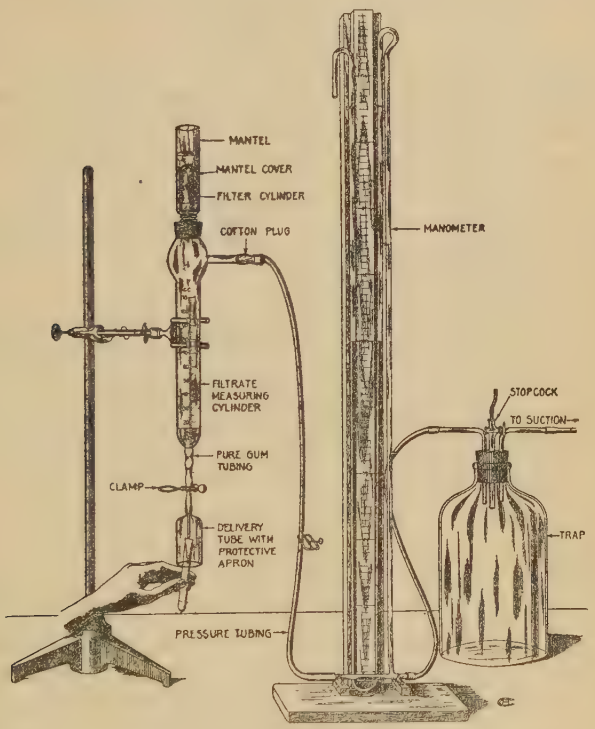

FIG. 1.

working with *V. percolans* that the first portions of the filtrate may be sterile, while later portions contain organisms, it is obvious that a control of the stages of filtration is highly desirable.

Effect of Physical Factors.—Filtration is the passage of a liquid containing suspended particles through capillaries. A discussion of the effects of physical factors on filtration, therefore, involves a consideration of their effect (1) on the flow of the suspending liquid, and (2) on the

suspended particles. The rate of flow of liquids through a straight capillary tube has been formulated in the law of Poiseuille:

$$V = \frac{\pi\, gpR^4t}{8\, l\, \eta}$$

V is the volume of fluid flowing in time, t; p is the pressure in gms. per sq. cm., and g the acceleration due to gravity; R is the radius of the capillary tube; l is the length of the tube; η is the viscosity of the fluid at the temperature of the experiment (34).

Although the capillary channels through bacterial filters are tortuous and non-uniform, an approximate proportionality of volume flow and filtration pressure when a pure liquid is passed through the filters has been observed by a number of experimenters, Marchoux (105), P. Schmidt (154), Mudd (119). Under conditions of actual work, however, the pore radius is continually being reduced by clogging so that V decreases with time. Curves showing this effect have been published by P. Schmidt (154) and others.

Concerning the effects of physical factors on the particles in the filtering fluid so little has been done that it is scarcely worth discussing. There is evidence, however, that microörganisms which are retained at low pressures may sometimes traverse filters at high pressures (80, 149, 164). The plugging of filters proceeds more nearly to completeness at high pressures, however, because particles are forced into the pores (105, 75). Particles are reputed to appear more quickly in the filtrate with intermittent than with constant pressure (19, 161). When a plastic particle is forced through a capillary smaller than its cross-section (24) a certain critical pressure must of course be exceeded.

Adsorption.—Failure of a virus to appear in the filtrate does not warrant the conclusion that it is too large to pass the filter pores, unless the possibility of its retention due to adsorption has been excluded. The adsorption of certain substances has been reduced or eliminated by preliminary passage through the filter of egg white or peptone (83), of heated serum (128), or of oil (85). The danger of clogging the filter by an excess of these substances must be remembered.

Substances that are desired to pass through siliceous filters with a minimal loss should be filtered at reactions more alkaline than their isoelectric points. A compilation of reported isoelectric points has been made by Mudd (120). The danger of alteration of labile substances at too alkaline reactions (47) should be borne in mind.

Cleaning Filters.—There are many ways of cleaning filter candles.

The following method, which is essentially the one recommended by the manufacturers of the Mandler filter, is useful:

If infectious material has been filtered, first immerse the candle in a non-coagulating germicide long enough to effect sterilization. Remove débris from outside of candle with a stiff hand brush, avoiding rough handling. Place candle in a metal holder or connect it with such a device as described by Krock and Holman (94). Wash from within outward under pressure not exceeding 25 lbs. per sq. in. If the filter pores contain globulin, as after filtration of serum, 0.85 per cent NaCl should be used for washing; otherwise water will do. Washing should continue until all visible impurities are removed. Boil candle in 2 per cent solution of Na_2CO_3 for $\frac{1}{2}$ hour. Boil for 1 hour in several changes of distilled water. Again wash the candle from within outward with water until all alkali and organic impurities are removed, brushing surface if necessary. Retest candles by the air-pressure method.

Protocols.—In the studies on the filterability of active agents there should be recorded, in addition to full biological details, any preliminary manipulation of the filter or of the material to be filtered; the type and size of filter used; whether filter is new or old, and, if used, for what purpose; air-pressure test of filter; content and dilution of fluid to be filtered, including active agent, control organisms, colloids, electrolytes, and approximate reaction; filtration pressure; volume of filtrate; the stage in the filtration process at which specimens are collected for tests; media and animals into which specimens of the filtrate are inoculated.

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TISSUE CULTURES IN THE STUDY OF VIRUSES

ALEXIS CARREL

CHAPTER III

TISSUE CULTURES IN THE STUDY OF VIRUSES

INTRODUCTION

No lifeless culture medium has as yet been discovered which provides a virus with the substances required for its maintenance and growth. It is possible that such a medium will never be found because some viruses may be non-living substances manufactured by tissues or blood cells, and not ultramicroscopic organisms. So far, a virus has always required the presence of living cells in order to propagate itself. Within the animal body, it generally inhabits the tissues rather than the humors. Its affinity for living cells indicates that, whatever may be its nature, it must propagate in tissues outside as well as within the organism. Through the rudimentary techniques of the early days of tissue culture, the agents of poliomyelitis (29), rabies (30), and vaccinia (35, 36, 14, 15) were found to persist in fragments of tissues as long as the cells remained alive. Thus, it was demonstrated that tissues kept *in vitro* can be utilized in the investigation of the properties of viruses. Although fourteen years have elapsed since this work was undertaken, the method of tissue culture has not greatly increased our knowledge of the heterogeneous group of filterable pathogenic principles. This must be partly attributed to the fact that pathologists are far from having mastered the techniques for the cultivation of tissues. Most of them have used the comparatively crude procedure which was derived immediately from the experiments of Harrison (16), and consisted in embedding a fragment of fresh tissue in a drop of plasma on the coverglass of a hollow slide. The cultures were composed of mixtures of cells of different types in an active or degenerate condition, surviving in a medium that spontaneously underwent profound modifications in its composition. No important discovery could be expected from such a technique. On the contrary, it is certain that the modern methods, by which pure strains of given cell types are cultivated in flasks in a medium of known composition and maintained in a condition of measurable activity for long periods of time, will help to ascertain the nature and fundamental properties of viruses. A virus may be a very minute organism, or it may be a chemical substance manufactured by the cells themselves. In

either case, its multiplication depends upon the activity of a living tissue. Since pure strains of cells are caused to live *in vitro* under such simple conditions that the effects of viruses on cell morphology and reactivity and the relation of the nature and metabolic condition of cells to the viruses may be analyzed, new discoveries will certainly be made. It is obvious that the cultivation of tissues, in its modern form, reduces to very simple terms the problem of the relations of virus and cells, whatever the living or non-living nature of the agent may be. But its success in the investigation of these complex phenomena depends almost entirely on the acquisition by pathologists and bacteriologists of the mass of technical knowledge which renders precise the handling of living tissues *in vitro*.

Technique

Selection of the Tissues and Their Inoculation with the Virus.—The tissues must originate from an animal susceptible to the disease of which the causal agent is being studied. When a given virus is able to propagate in several types of animals, the one having tissues that may be easily cultivated *in vitro* should be selected. The tissues of chickens, mice, and rats are especially suited for these studies. But those of guinea pigs, rabbits, monkeys, and human beings can also be used. According to the aspect of the problem under investigation, fragments of embryonic or adult tissues recently extirpated from the body of the animal, or pure strains of cells of various types, are employed. Fresh tissues, such as skin, brain, spleen, heart, bone marrow of adult animals or embryos, and blood leucocytes, are excellent substrata for the growth of viruses. When the purpose of the experiment is simply to manufacture a virus *in vitro*, leucocytic film, fragments of bone marrow, and embryonic pulp are suitable. The bone marrow of the femur of a guinea pig, or the pulp from an 8-day chick embryo furnishes an amount of tissue sufficient for the production of a large quantity of the agent. But if the subject of the investigation is the study of the relations of a virus to a given tissue, such a technique is too crude. In cultures composed of cells of various types, the part played by each type in the production of the virus cannot be ascertained accurately. Besides, the relative proportions of the different cells vary widely from day to day. For instance, the cultures composed of fragments of cornea, brain, or embryo pulp contain a large number of epithelial cells and fibroblasts during the first few days. Later, the epithelial cells disappear upon the invasion of fibroblasts. It is then impossible to know

the constituents of the tissue exactly, or to ascertain their relative activity. The same phenomenon occurs in cultures of spleen and bone marrow, which consist of ameboid cells and fibroblasts. These tissues become modified according to the nature of the medium in such a manner that one or another of the cell types may completely disappear. Therefore, pure strains of cells must be used when the relations of a tissue to a virus are to be elucidated.

The making of pure cultures requires a precise knowledge of the technique. While no apprenticeship is needed for embedding a fragment of tissue in a drop of plasma, or even for cultivating a large amount of embryonic pulp in a flask, the isolation and maintenance of pure strains of fibroblasts, epithelial cells, or macrophages demand the mastery of a number of delicate procedures. These procedures are not difficult, but they have to be learned. Fibroblasts are obtained in pure culture from practically any embryonic or adult tissue of chickens, rats, dogs, monkeys, or men. They grow more rapidly than any other cells in media composed chiefly of embryonic tissue juices or of proteoses. In such fluids the ameboid cells multiply more slowly than do the fibroblasts and disappear in a few weeks. Epithelial cells are progressively replaced by the more rapidly proliferating fibroblasts. Ultimately, the cultures are composed exclusively of fibroblasts which remain unmodified for indefinite periods of time. Pure cultures of epithelium are more difficult to obtain. The cells have to be isolated mechanically from the fibroblasts. Pavement epithelium of the iris, and thyroid cells have been kept in active condition for months and years. Malpighian epithelium, lens cells, etc., have also been obtained and grown in pure state. Macrophages are easily isolated from the blood. The layer of white cells which covers the red blood corpuscles of centrifuged blood is coagulated by a few drops of tissue extract, removed from the tube, and cut into small rectangular fragments. The fragments are then embedded in the solid medium contained in a flask, and the leucocytes migrate. After 1 or 2 weeks, the polymorphonuclears, the lymphocytes, and the red blood corpuscles have disappeared, a great many of them having been phagocyted by the monocytes. Thus, a pure culture of macrophages is obtained. Strains of these cells may also be isolated from spleen and from fragments of adult connective tissue.

The technique of inoculating the tissues with a virus varies according to the nature of this agent. The simplest procedure consists of injecting the virus directly into the flask containing the tissues, fresh fragments, or pure strains of cells. This practice generally succeeds when the virus

is not rapidly destroyed by the medium. For instance, a few drops of filtered extract of Rous sarcoma injected into a flask containing macrophages are generally sufficient to produce the inoculation of the cells. But if the virus is more susceptible to the medium, its inactivation may occur before it has been in contact with the tissues for a sufficient time to inoculate them. Therefore, a better procedure consists in placing the flasks in the refrigerator after they have been injected with the virus. After from 1 to 6 hours, the flasks are returned to the incubator. This generally gives the virus time to penetrate the medium and reach the tissues before becoming inactivated. It seems, however, that the inoculation of the tissues with the virus prior to the preparation of the culture is more generally successful. The fragments of tissues or of pure strains are deposited in tubes containing the virus suspended in high concentration in Tyrode solution having a pH properly adjusted. Then the tubes are kept in the refrigerator for a period lasting from 1 to 24 hours. For the viruses of vaccinia and Rous sarcoma, 1 hour is enough. When a culture must be inoculated with a fragment of tissue already infected, the procedure is much simpler. It is sufficient, under these conditions, to cultivate the infected tissue and the normal tissue side by side in a flask. After a short time, the agent propagates itself within the normal cells.

The Flasks.—The tissues are cultivated in flat round flasks of three diameters, 3 cm., 5 cm., and 8 cm. They belong to our Type D culture flasks, which consist of a round container 1 cm. to 1.5 cm. high, having an oblique neck 1 cm. in diameter and 3 cm. long. The obliquity of the neck allows the frequent opening of the flask without danger of bacterial infection. The dust that may fall from the air remains adherent to the glass, and is easily destroyed by flaming. Then the flask is closed with a rubber cap and a little non-absorbent cotton. The cap must fit the glass tightly in order to prevent evaporation of the fluid and escape of carbon dioxide. Some other types of flasks have been made with openings at the top or bottom, or with two necks, but they have not proved as convenient for this special purpose. It is essential that these flasks be regular in form and of a good quality of glass which does not set free any alkali. If the bottom is not flat, the thickness of the medium is uneven, and the density of the colonies becomes irregular. The transparency of the bottom is sufficient to allow examination of the tissues at a magnification of about 100 diameters. This simple type has been used a great many times during the past few years and infection was found to occur in less than 1 per cent

of the cultures. The modifications of this type advocated by other experimenters or tried by ourselves so far have not been an improvement. This is especially true of Borrel's containers with a horizontal neck, which deprives the flasks of almost all their value.

Preparation of the Cultures.—The medium is composed of two parts, one solid and the other fluid. The solid part consists of diluted plasma in which the tissues are placed before coagulation. A fibrin coagulum is the best scaffold that we possess for cell migration and multiplication. Fibroblasts and macrophages grow inside the clot, while the epithelial cells generally spread on its surface. The transparency, adherence to the glass, contractility, and friability of the coagulum obtained from the plasma of the different animals used in laboratories vary greatly. Chicken blood furnishes the best medium because its plasma remains clear, does not contract, and allows the cells to multiply for a long time without need of repairing the coagulum. Fibroblasts grow from 7 to 15 days, and macrophages from 15 to 30 days, before it is necessary to transfer them into a new flask. Guinea-pig and rat plasmas, although much inferior to chicken plasma, have the proper consistency and transparency. Coagulated rabbit plasma rapidly becomes opaque and contracts into a thin layer on losing its serum. Human and monkey plasmas produce a coagulum which liquefies rapidly under the influence of the tissues. Chicken plasma yields a better coagulum than any other, even for the cultivation of mammalian tissues. The toxic effect of serum on foreign cells is prevented by washing the coagulum with Tyrode solution. The serum remaining in the clot after this procedure is of such a weak concentration that it has no poisonous effect on the tissues. In practically every flask, the medium is prepared in the following manner: One volume of plasma is mixed with 3 or 5 volumes of Tyrode solution at pH 7.5. Two cc. of this mixture and 3 drops of diluted extract of embryonic pulp are injected with a pipette into D-5 flasks. When a D-3 flask is used, the volume of the medium is only 1 cc. Then the fragments of pure culture or of fresh tissue are introduced with a platinum spatula or a pipette. After a few minutes, coagulation takes place. The clot is then washed twice, or not washed, depending on whether homologous or heterologous cells are used. The washing consists of introducing into the flask from 3 to 10 cc. of Tyrode solution, and removing it after 15 minutes.

The preparation of the culture is completed by the introduction of the nutritive fluid medium on the surface of the coagulum. The amount is 1 cc. for D-5 flasks and 0.5 cc. for D-3 flasks. The composition of the

fluid varies according to the nature of the tissue. Strains of epithelial cells and fibroblasts are fed upon diluted embryonic juice from the pulp obtained by passing chicken, mouse, and rat embryos through a Latapie apparatus. As embryonic tissue juices from animals of widely different species, such as chickens, rabbits, and rats, can be used indiscriminately by fibroblasts and epithelial cells for the building up of protoplasm, chicken juices are generally selected as a nutritive medium, because they are easily obtained aseptically. The primary split products of Witte's peptone, ox fibrin, or organs such as calf pituitary gland or liver, may be substituted, in some measure, for embryonic juices. The proteoses bring about a large growth of epithelial and mesenchyme cells. On the contrary, plasma or serum not only fails to be nutritive for fibroblasts and epithelial cells, but also markedly inhibits their proliferation. Pure strains of macrophages from the blood or connective tissue, and fresh bone marrow and spleen fragments require serum or plasma as the chief constituent of their nutrient medium. Since the discovery of heparin by Howell (19–21), plasma can be used in the fluid state. If the surface of the coagulum has been thoroughly washed, diluted plasma containing about 1/10,000 of heparin does not coagulate in the incubated flasks for several days. Serum or plasma is the optimum medium for the growth of ameboid cells.

Cultures of epithelial cells and fibroblasts are washed every 2 days, and those of macrophages every 3 or 4 days in 3 or 6 cc. of Tyrode solution, according to the size of the flask, and fresh nutritive medium is added. If the clot begins to be digested, it is reinforced with a little plasma and embryonic juice. When the growth of the colonies of fixed cells becomes less active, or after the complete invasion of the flask by the leucocytes, the tissues are transferred to another container. This operation is carried out very easily with a platinum spatula. The clot is first detached from the glass near the base of the neck by means of a straight, flexible spatula. By pressure of its tip on the wall of the neck, the proper curvature is given to the instrument and the clot is then detached from the circumference of the flask and from all its surface. The clot is removed and spread on a sterilized glass plate where it is cut with a cataract knife into rectangular pieces. These fragments are placed in other flasks for the continuation of the experiments, or on coverglasses for examination with the immersion lens, or they are ground in a mortar for inoculation of the virus into animals.

Measurement of Cell Activity.—The activity of a tissue may be measured by the increase of its mass or by the amount of oxygen absorbed

and of carbon dioxide set free. When the metabolism of large fragments of tissues, like flaps of skin, is being studied, the Warburg method for measuring the amount of oxygen absorbed may be used, as Rhoda Erdmann and Schmerl (12) have done. However, this is not possible when pure strains of cells are concerned. On account of the small volume of the tissue and the presence of fibrin in the coagulum, the weight of the cells cannot be measured. Generally, the activity of a colony is ascertained by its rate of growth. The flask is placed in a projectoscope and the outline of the tissue is drawn immediately after the preparation of the culture and again after 48 hours of incubation. The area of the original fragment and of the new growth are measured with a planimeter. The ratio of the area of the new tissue to that of the original fragment, or relative increase of the colony during 48 hours, expresses the rate of growth. As the growth energy of a tissue depends on several factors, each experiment must be controlled by a fragment of the same tissue under standard conditions, and the rate of growth of the experimental fragment is expressed as a function of the rate of growth of the control. The ratio of the relative increase of the tissue in the experiment to relative increase of the control tissue is called the growth index. The growth index of a given tissue is an accurate expression of its proliferative activity. It is far more difficult to ascertain the activity of colonies of ameboid cells because they do not grow as a tissue, but scatter themselves through the medium on several planes until the flask is entirely invaded. Under these conditions, the time taken by the cells to cover the entire surface of the solid medium may be considered as the expression of their activity.

Contribution to the Knowledge of Viruses through Tissue Cultivation

This contribution (31) has so far been meager, as the method of tissue culture has been employed chiefly to confirm some well-known facts already observed in living animals, such as the presence and the multiplication of viruses within the tissues. Long ago, viruses were found to persist in tissues surviving *in vitro*. This was shown in 1913 by Levaditi when he succeeded in maintaining the virus of poliomyelitis (29) in active condition for several weeks in spinal ganglia "cultivated" in plasma. The virus of rabies (30) was also found by the same author to be capable of surviving more than a month in rabbit medulla. In the same year, Steinhardt, Israeli, and Lambert found that vaccine virus (35) retains its activity for more than a month in fragments of cornea surviving in a drop of plasma. *Rickettsia prowazeki* was studied

in vitro by Kuczynski (24–28), Wolbach and Schlesinger in 1923 (37), and Krontowski and Hach in 1923–24 (22, 23). It was thus demonstrated that the etiological agent of typhus fever remains active for a long time in tissue cultures. The virus of Rocky Mountain spotted fever was also studied in tissue culture by Wolbach with Schlesinger (37) and with Pinkerton (37, 39), who maintained it in a condition of activity for 28 days. The experiments of Rhoda Erdmann (10, 11) showed that an abundant production of the virus of fowl plague takes place in cultures of bone marrow. It was also observed that the virus of Rous sarcoma multiplies in cultures of leucocytes or embryonic pulp (1). Not only do viruses survive in tissue cultures, but they increase markedly in quantity. Parker alone (32) and with Nye (33) inoculated fragments of testicles with vaccine virus and cultivated them in plasma for a long time. They found that the tissues of the eleventh generation of the culture contained 51,000 times as much virus as the first cultures. From these experimental data, it may be concluded that viruses multiply in tissues living *in vitro*, and that they disappear from the cultures when the tissues die. Although these findings do not extend our knowledge of the viruses, and are merely the expression of some of their well-known properties, they are, nevertheless, of great interest because they show that tissue culture can be applied to many problems concerning the production of viruses, and the nature of their fundamental characteristics.

Since viruses multiply in small fragments of tissue living *in vitro*, we may take advantage of this property in order to manufacture large quantities of these active agents in a pure state. Cultures of embryonic pulp and leucocytes in D-5 flasks inoculated with filtered extract of Rous sarcomas have been used for the routine fabrication of Rous virus. For several months, each flask produced at least 1 cc. of highly virulent fluid every day (7). The same procedure has been applied recently to the manufacture of vaccine by Rivers and myself (8). Pulp made of skin, cornea, or brains of chick embryos, or of the whole embryo, was inoculated with vaccine virus obtained from the testicle of a rabbit. A known quantity of the pulp was suspended in a definite quantity of Tyrode solution containing the virus. After the suspension had stood for some time in the refrigerator, the cultures were prepared in D-5 flasks in the ordinary way. Every 2 or 3 days, they were washed with Tyrode solution and fed with diluted extract of chick embryos. The embryonic pulp which was originally inoculated with 25 or 250 units per cc. contained from 10,000 to 100,000 units per cc. after 8 days' cultivation. It is probable that a chick embryo crushed to a fine pulp

is capable of producing as much vaccine as a calf. The technique is simple and is susceptible of adaptation to the industrial production of vaccine virus, when an absolutely pure product is needed.

Very few attempts have so far been made to analyze by means of tissue culture the process of virus formation, and the fundamental relations existing between the tissues and the pathogenic principles. However, some workers have undertaken the study of certain aspects of this complex problem. Steinhardt and Lambert (36) have investigated the effect of the metabolic activity of the tissues and of immunity on the growth of vaccine virus. The action of vaccine virus on corneal cells and the production of vaccine granules were studied by Craciun and Oppenheimer (9). However, the techniques used by most of the experimenters were not sufficiently perfected to allow a precise understanding of the phenomena.

The more efficient modern techniques were applied for the first time in 1924 to the study of viruses when the effects of filtered extract of Rous sarcoma on pure cultures of macrophages and of fibroblasts were analyzed (1). These experiments were made with a view of ascertaining which cell type is malignant, that is, possesses the property of carrying and possibly of manufacturing the virus. When a culture of fibroblasts was inoculated with Rous virus, the cells generally did not undergo any change, and the virus rapidly disappeared from the fluid in the flask (2). On the contrary, cultures of macrophages inoculated in the same manner often digested their medium (1), and almost always caused a large amount of virus to appear in their fluid medium. However, the virus may also multiply in cultures of macrophages which do not show any morphological change. It became evident from these experiments that macrophages are far more susceptible to Rous virus than fibroblasts are and that, although Rous sarcoma is a spindle cell tumor, the malignant element is the macrophage and not the fibroblast. This result could not have been obtained by any other method than that of tissue culture in its modern form. The study of infected animals, or even of fragments of fresh tissues surviving *in vitro*, does not allow the elucidation of the nature of the cells responsible for the growth of the virus. For instance, when poliomyelitis virus was cultivated in fragments of spinal ganglion by Levaditi (29), and the virus survived in spite of the degeneration of the nerve cells, no precise conclusion could be reached regarding the nature of the cells required by the virus for its proliferation. The only manner of determining exactly whether mesenchyme or nervous cells cause the multiplication of the virus would be its cultivation in a pure strain of fibroblasts.

It has also been possible to determine the quantity of tissue which is required to produce a definite amount of virus (7). We have found that the smallest amount of spleen which permits Rous virus to persist in a D-5 flask containing the usual quantity of culture medium is approximately 1 c.mm. of tissue in 3 cc. of medium. If the amount of tissue is made from 5 to 9 times larger, an abundant production of virus is always obtained. The quantity of active virus present in a flask containing multiplying cells depends chiefly upon the volume of the tissues. Although tissue culture has not as yet contributed any new and important knowledge on the nature or the properties of viruses, and has only confirmed the conceptions based on the observation of living animals, it has, however, proved that it is an excellent instrument for future investigations of problems relating to virus diseases.

The Value of Tissue Culture in the Future Investigation of Viruses

There is no doubt that the growth of viruses in pure strains of the main cell types living in a given metabolic condition within a medium of definite composition will lead to the discovery of some of their properties. When studied in the animal, the problem of the relations of viruses to tissues and humors of the body is exceedingly complex. It would be rendered relatively simple, however, if it were made to consist merely of the reciprocal influence of a virus upon one type of cells free from body fluids. We may consider as probable that a close analysis of these relations would lead to a division of the viruses into two classes: ultramicroscopic organisms, and non-living chemical principles analogous to the lytic substance of Twort or bacteriophage of d'Herelle. The properties which distinguish living exogenous viruses from endogenous cellular viruses might thus become apparent if the nature of their relations with the tissues were more closely analyzed.

The behavior of each virus must be studied in pure cultures of tissues and blood cells, as has been done with Rous virus. The relations between the nature of the cells, their quantity, their metabolic activity, and the quality and quantity of virus produced can be investigated with the assistance of this method. It is not unreasonable to believe that a virus, growing in cells of various types, may acquire quite different properties. Such a problem cannot be attacked by experiments conducted on living animals, or even by the cultivation of tissues according to the old techniques. Its investigation requires, on the contrary, the use of the most elaborate of the new procedures. Thus, it may become possible to ascertain whether the viruses which are supposed to be the

product of cell activities are related in a more intimate manner to the quality, quantity, and metabolic activity of the cells than are the viruses consisting of ultramicroscopic organisms. Through similar methods, the modifications imposed upon the cells by the presence of the virus could be analyzed more accurately than has been done so far. The changes that each virus may bring about in the morphology and the reactivity of each cell type must be investigated. Such a study has already been made with pure cultures of fibroblasts and macrophages, from the point of view of the effect of Rous virus. Certain viruses probably have the property of growing without modifying the cells which they use as a substratum, as happened with macrophages in the presence of Rous virus. Other viruses will, without doubt, produce characteristic lesions, such as have been observed in the tissues of living animals. It is possible that, according to the living or non-living nature of the virus, the cytological changes in the cells will assume profoundly different characteristics. At the same time, it will be easy to observe how the reactivity of a given strain of cells is modified by a virus. We may suppose that the cells will become immune more readily against living viruses which are of a foreign origin than against a substance which is formed by the cells themselves. This problem and many others, which are of the greatest biological importance, offer a vast field of investigation, and no method of solving them is as obvious as that presented by tissue culture.

At the same time, these techniques give us the best means of approaching the capital problem of the artificial production of a virus by the action of chemicals on cells. Until a virus can be manufactured *in vitro* in such a manner, we will remain ignorant concerning the nature of many pathogenic agents which are not living microörganisms. The injection into chickens of embryonic tissues and certain chemicals, such as arsenic, coal tar, indol, and peptone, produces sarcomas which contain the Rous virus (3). This fact immediately suggested that Rous virus is not a microörganism, but a product of cell activity. In attempting to determine the occurrence of this phenomenon *in vitro*, I found that cells treated with arsenic may become modified. They acquired and retained the property of digesting the medium for many generations. In similar experiments, by treating a fragment of spleen with arsenic *in vitro*, Fischer (13) succeeded in obtaining a malignant sarcoma, which in turn was found to produce the Rous virus. It seems as if tissue cells might undergo a mutation due to the effect of certain chemicals, and acquire some definitely new properties among which are

those characteristic of malignancy. A precise knowledge of the physico-chemical conditions which bring about such important changes may possibly be acquired by experiments conducted on tissues living *in vitro*. In this manner, the method of tissue culture may lead to the solution of some of the most important biological problems of the day.

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INTRACELLULAR PATHOLOGY IN VIRUS DISEASES

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CHAPTER IV

INTRACELLULAR PATHOLOGY IN VIRUS DISEASES

One of the most striking attributes of many diseases caused by filterable and as yet ultravisible viruses is the development of large and conspicuous "inclusion bodies"[1] within the cells. Some of these are represented in Plate 1 in the typical colors which they assume after staining. They may occur in the cytoplasm, in the nucleus, or in both. There exists in addition a fourth group of bodies some of which have been erroneously linked with the filterable viruses, while the status of others is either suspected or wholly unknown. We shall consider them in the following order:

Cytoplasmic inclusions:[2]
- Vaccinia
- Sheep-pox
- Rabies
- Molluscum contagiosum
- Infectious myxomatosis of rabbits
- Lymphocystic disease of fish

Nuclear inclusions:[3]
- Herpes
- Varicella
- Virus III infection of rabbits
- Salivary-gland disease of guinea pigs
- Visceral disease
- Borna disease of horses

Cytoplasmic and nuclear inclusions:
- Smallpox
- Paravaccinia

Inclusions of doubtful nature:
- Warts
- Mucous papillomata
- Psoriasis
- Carp-pox
- Swine fever
- Epidemic encephalitis
- Nervous distemper of dogs
- Fowl plague
- A disease of fowls in Nigeria
- Kurloff bodies in guinea pigs
- Todd bodies in erythrocytes
- South African horse sickness

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[1] The term "inclusion body" has no descriptive value and is perhaps unfortunate because it conveys the impression that the structures so designated are in some way "included." Yet it is applied with equal freedom to materials developed within the cell and to others obviously taken in from without. It is so loose a term that it really means nothing at all and carries no distinction between the bodies and droplets of fat, ingested pigment, the remnants of phagocytized bacteria, and many other materials. The designation x-body employed by botanists (*cf.* Goldstein, B., *Bull. Torrey Bot. Club.*, 1926, liii, 499) is less open to objection and in my opinion merits consideration with a view to general usage for "inclusions" developed by these peculiar agencies until we learn more of their nature. We would thus have the x-bodies of vaccinia, herpes, and other diseases to con-

It is natural that there should be much divergence of opinion regarding the interpretation of these inclusion bodies. It arises from the tendency of investigators to place reliance in different kinds of evidence. While the viruses themselves continue to resist all attempts at cultivation on artificial media, and we continue to be ignorant as to whether we are dealing with living agents, the problem remains primarily a cytological one.

When the specific inclusions were first discovered, they were hailed more or less tentatively as the actual parasitic agents of the diseases, were grouped among the protozoa and were given generic and specific names of which *Cytoryctes vacciniae* and *Neurocytes hydrophobiae* will serve as examples. This was done at a time when contemporaneous studies on the malarial organism exercised a profound influence in the scientific world. Much emphasis was laid on progressive changes in morphology as indicative of the existence of life cycles. But closer and repeated scrutiny of the supposed life cycles did not turn out to be reassuring and the discovery that the viruses would pass through fine filters was difficult to reconcile with the belief that the relatively large inclusions were themselves the infective agents. A reaction set in and several papers appeared indicating that many of the inclusions may not in fact be protozoan parasites but complex deposits of material never before seen and defying chemical analysis. This modification in point of view developed coincidently with a fuller appreciation of the ability of cells to adapt themselves to unusual conditions, in other words, that they are not fixed in structure but may respond in all kinds of peculiar ways to environmental changes.

A compromise hypothesis was accordingly advanced by v. Prowazek (56). According to his conception, the inclusion bodies are neither protozoa nor pathologic deposits but complex structures of dual nature consisting of microörganisms imbedded in material produced by the cell in response to their action. He proposed that the microörganisms be grouped in a new class which he called "Chlamydozoa"—literally, mantle

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sider. This would make for simplification of terminology and would be more rational.

[2] The cytoplasmic inclusions typical of trachoma are excluded; for the virus probably no longer belongs in this class of ultravisibles since Noguchi (51) has found that in Indian trachoma it is a bacterium. The mosaic diseases of plants are discussed in Chapter IX and contagious epithelioma (fowl-pox) in Chapter VII.

[3] The polyhedral diseases of insects are considered in Chapter VIII and foot-and-mouth disease and vesicular stomatitis in Chapter VI.

animals—to indicate this habit of being clothed in a mantle of cellular substance. In his opinion, the chlamydozoa are typically intracellular parasites which differ from bacteria in their method of multiplication as well as in other respects. He conceived their life history to be somewhat as follows: In the stage of "elementary corpuscles" they are extracellular and filterable. Soon after penetration through the cell membrane they may be detected in the form of tiny "initial bodies." These initial bodies become coated with cellular material, grow, mature, and break down with the liberation of large numbers of the original elementary corpuscles, which, in turn, repeat the process by entering and infecting neighboring cells. The term *Strongyloplasmen* has been devised by Lipschütz (35) to indicate organisms endowed with much the same properties.

This view has not proved entirely satisfying. Many believe that the bodies are neither organisms *sui generis* nor combinations between organisms and cellular components but rather *reaction products* produced by the cell in response to injury caused by infective agencies which are ultramicroscopic. Weight is placed in two principal considerations:

First, lack of definite heterogeneity on the part of the inclusions. A good deal of misunderstanding is to be attributed to reliance in fixed preparations which, owing to artificial coagulation, and exaggeration of contrasts caused by methods of coloration, often convey a false impression of heterogeneity. On careful cytological analysis, some of the inclusions appear to be amorphous and quite devoid of visible internal organization, while others are composed of several constituents which only in the minds of a few investigators can be interpreted as organismal in nature.

Second, failure to confirm the descriptions of v. Prowazek and others relative to the existence of "elementary corpuscles" (Paschen's corpuscles) in infective blood and filtrates known to contain the viruses. This has come about by an appreciation of how closely inanimate protein and other substances, when finely divided, will simulate microörganisms in appearance and by improvements in the dark-field microscope and ultramicroscope.

Yet advocates may be found among experienced workers for all three of the above mentioned theories. This is after all to be expected because just as individuals value the same evidence differently in terms of their experience and point of view so, also, the viruses and inclusions are different and will doubtless in time be explained in different ways. The subject has suffered from attempts to formulate hypotheses of wide ap-

plication. Each disease is in itself a separate problem and will here be so treated, briefly, at the risk of repetition of features possessed in common. Only the cytology will be stressed. Other considerations are discussed in detail elsewhere. From the cytological point of view, the chief questions which arise and which can now only be answered in the most fragmentary way are the following:

1. What is the nature of the material of which the inclusion bodies are composed?
2. Is such material present within the cell prior to the action of the virus?
3. What is the relation between the developing inclusion bodies and other recognizable cellular constituents?
4. What alterations in cellular activity are caused by the viruses?
5. Do the viruses act on the surface of the cell or penetrate into its substance?
6. Can the inclusion bodies be produced by agencies other than the viruses?

CYTOPLASMIC INCLUSIONS

Vaccinia

(Guarnieri bodies, *Cytoryctes vacciniae*, vaccine bodies)

Vaccine bodies as they appear after fixation in Zenker's fluid and coloration with eosin and methylene blue are illustrated in Plate 1, Figs. 16 and 17. They may be seen as irregular pink and lilac masses which indent the nuclei. Of all the pathognomonic inclusions, they lend themselves best to experimental study. They may be produced at will, on only a few hours notice, in the corneal cells of the rabbit by simply making a slight scratch and adding the virus. It is instructive to

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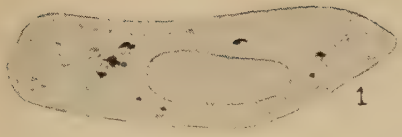
FIGS. 1–5. Cells of a rabbit's cornea supravitally stained with brilliant cresyl blue. 1. An unvaccinated cell showing traces of the material of which the vaccine bodies are built. 2. A larger amount of similar substance developed under the influence of the virus. 3. The material becomes heterogeneous, dark droplets (colored blue in the living state), and unstained vacuoles being now visible. 4. A much larger vaccine body with smaller ones clumped about it. 5. A cell containing vaccine bodies (nucleus not represented) which has phagocytized another cell. × 1500. (After Cowdry, 1922.)

FIGS. 6–10. Cells of a rabbit's cornea fixed in Zenker's fluid and colored with Giemsa's stain. 6. An unvaccinated cell exhibiting traces of material like that forming the vaccine bodies. 7. More material of the same kind developed as a result of infection by the virus. 8. A vaccine body closely applied to the nuclear membrane containing three uncolored areas. 9. Vaccine bodies indenting a nucleus. 10. A cell possessed of vaccine bodies which phagocytized another cell. ×1500. (After Cowdry, 1922.)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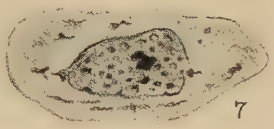
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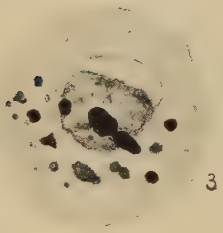
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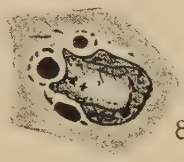
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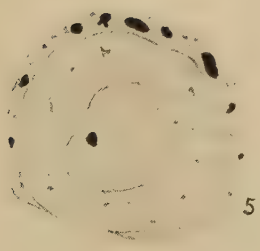
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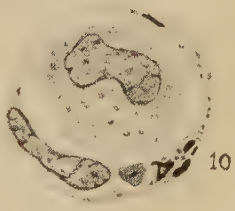
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follow the changes which they undergo as viewed in the living state and in fixed and stained preparations.

Figs. 1–5 represent diagrammatically stages in the formation of vaccine bodies in corneal cells supravitally stained with brilliant cresyl blue, and Figs. 6–10, roughly parallel stages in cells fixed in Zenker's fluid and colored by Giemsa's method.

Both series begin with cells (Figs. 1 and 6) which have not been subjected to the action of the vaccine virus yet show in their cytoplasm traces of the material of which the vaccine bodies are built. This is colored pink in the living condition (Fig. 1) and blue in the fixed cell (Fig. 6). It is dark gray in the figures.

The process of fixation and staining gives in some respects an erroneous idea of conditions in the living state. It will be noticed that the cells are thereby definitely shrunken, but not so greatly as one would estimate from a comparison of the two series, for the living cells become somewhat flattened while under observation so that their apparent area, viewed from above, is increased. On the other hand, the fixed cells are more angular and thus more nearly approach their true shape when fitted together in tissue formation than do the isolated living ones, which, owing to the release of confining forces acting on their surfaces, become more spherical than they are in the living animal. Methods of fixation and staining often convey a false impression in regard to the vaccine bodies, and this discrepancy applies likewise to many of the other inclusions described in this chapter. The really clear-cut boundaries of the inclusions in living cells may be blurred by the fixation and subsequent treatment so that they seem to grade into the cytoplasm; and halos and clear-looking spaces are frequently formed either as an exaggeration of preëxisting structure or through differential shrinkage at interfaces.

More and more of this substance is formed under the influence of the virus and develops a definite heterogeneity but not of a kind to suggest a combination between invading microörganisms and cellular components. In the still living cells, this heterogeneity is evidenced by the elaboration within the pink-stained masses of droplets which are colored blue (black in Figs. 2 and 3) and of spherical areas which do not take any stain but appear clear (Fig. 3). In permanent preparations, the material comes to react in two ways to such stains as that of Giemsa. Most of it tends to be pink but parts of it are blue. In both series, the vaccine bodies may hug the nuclei (Figs. 3 and 8) or they may not. In the fixed cells, the vaccine bodies show a much more marked inclination to indent the nuclei (Fig. 9). Phagocytosis of one cell by another containing vaccine bodies is of common occurrence (Figs. 5 and 10).

It is difficult to define the relation probably existing between the

development of vaccine bodies and other recognizable cellular constituents. The cell acts as a unit and all of its parts are presumably concerned; but to assert that either the nuclei, nucleoli, centrosomes, or cytoplasmic reticulum play a dominant rôle in their manufacture is unsafe. Schütz (66) is in agreement with me (Cowdry, 14) that neither the mitochondria nor the Golgi apparatus are directly involved in the sense that they contribute material to the developing vaccine bodies.

The reactions of the vaccine bodies *in toto* without distinction as to their components are as follows; but, on the basis of such feeble information, no conclusions as to their chemical nature are justified:

1. In the living cell, they are colored brick-red by neutral red. They are colored greenish blue by Janus green in a concentration of 1:10,000. This color changes to pink and is lost altogether on reduction at the same time that a parallel change takes place in the coloration of other cellular constituents.
2. They do not yield a positive Millon's reaction, oxydase reaction or the Bensley-Macallum hematoxylin test for masked iron.
3. They are quickly dissolved by the application of 1.5 per cent acetic acid.
4. They are not blackened by osmic acid or stained by Sudan III.
5. Ultraviolet photography of vaccine bodies in living cells brings out a chromatin-like structure (see Park and Williams, 53).

What the relation may be between the granules isolated by MacCallum and Oppenheimer (45) by differential centrifugation to the vaccine bodies is difficult to say. These authors believe them to be "identical with those seen in tissues and smears from vaccinia and smallpox by Prowazek, Paschen, Hallenberger and others." "They occur singly or in small groups. . . . These impress one as about one-tenth the size of a chain of streptococci with the same number of elements." The particles are of uniform appearance and can be seen with both direct and oblique illumination. "They stain faintly with Löffler's methylene blue, are gram negative, do not stain with neutral red or trypan blue, stain blue or red with Wright's stain and take a fairly deep stain with carbolfuchsin."

Oppenheimer has washed the granules and has found them still to be infective (52) and, working with Craciun, claims to have been able to cultivate the washed granules in tissue cultures for many generations. The material used to infect the cultures was prepared as follows:

"We removed 0.1 cc. of the top layer of the whole virus after centrifugalization and added it to 0.9 cc. of sterile Locke solution. The granules were washed thoroughly by pouring off the supernatant fluid and adding fresh Locke solution. This was mixed, again centrifugalized, and the precipitated granules washed

twice again in a similar fashion. Before inoculating the granules into the tissue cultures, they were always diluted to their original concentration in sterile Locke solution."

It is questionable whether by this treatment all the virus, which might have been adherent to the granules, had really been washed away. One is inclined also to ask whether the dilution thus effected has been sufficient to prevent the carrying over of enough virus, perhaps quite unassociated with the granules, to infect. In connection with the latter possibility, certain experiments by Parker and Nye (54) may be recalled. These investigators succeeded in definitely infecting tissue cultures by addition of the blood plasma of an infected rabbit in which the virus must be present in extremely minute quantities because other workers have uniformly failed to secure positive takes on the cornea and skin with it. In other words, negative corneal and skin reactions are compatible with the occurrence of traces of virus.

Oppenheimer freely admits that there is "no morphological proof of an increase in the number of the granules, since they cannot readily be distinguished from other granules normally seen in tissue cultures." The important point seems to be not merely to correlate the granules with elementary corpuscles of v. Prowazek and Paschen, which are of doubtful significance, but with the intracellular vaccine bodies which are unquestionably pathognomonic. When MacCallum and Oppenheimer first published on the subject, I attempted to do this (Cowdry, 14) and failed. Further work along this line is needed.

Sheep-Pox

The specific inclusions in epidermal cells affected by sheep-pox have never been studied extensively. In some respects they resemble vaccine bodies. They are microscopically heterogeneous as may be seen by reference to Plate 1, Fig. 22, which has been reproduced in the original colors, after staining with carbolic magenta-picro-indigo-carmine, from a paper by Bosc (9). The inclusion is situated to the left and slightly below the oval vesicular nucleus. The heterogeneity is, however, not of the kind one would take to indicate a combination between an invading organism and some cellular component. The cells which contain the inclusions are often actively phagocytic. For further information see Bosc (9) and Borrel (8).

Rabies

(*Neurocytes hydrophobiae* Williams and Lowden, Negri bodies, lyssa bodies, Lentz bodies)

An original illustration of a Negri body by Negri is reproduced in Plate 1, Fig. 21. It is evidently a definite structural element which, so far as is known, is not produced in any other condition except rabies.

More insistently than in the case of any of the other inclusions the view is advanced that the Negri bodies are true parasitic microörganisms probably protozoal in nature. This was Negri's (50) original contention. Williams and Lowden (74), on the basis of extensive studies,

FIG. 11. FIG. 12. FIG. 13.

FIGS. 11–13. Three cells containing parasites which, as one passes from left to right, tend to clump together to form, in the opinion of Manouélian and Viala, typical Negri bodies. (After Manouélian and Viala, 1924.)

reached the conclusion that they are microsporidia—a conception independently advanced by Levaditi, Nicolau, and Schoen 18 years later (33, 34). Levaditi and his associates were influenced by the analogy which they thought to exist between the Negri bodies and the so-called pansporoblastic stage of *Encephalitozoön cuniculi*. Levaditi believes that by treatment with hydrochloric acid and subsequent staining the Negri bodies may be resolved into masses of extremely minute "almost ultramicroscopic" granules, or microsporidia. Essentially the same conception is held by Manouélian and Viala (47). Several illustrations by Manouélian and Viala are reproduced in Figs. 11–13. They are

intended to show, passing from left to right, the agglutination of individual parasites to form typical Negri bodies. The parasites, judging by their morphology as represented, are so large and clearly organismal, that, if they are constantly found in rabies, it seems strange that they have not been reported earlier. It is possible that Manouélian and Viala were dealing with a double infection of rabies plus another organism, an encephalitozoön or a bacterium. The Negri bodies are referred to as organisms by Park and Williams in the eighth edition of their book (53).

These views rest wholly on the microscopically visible internal organization or heterogeneity of the Negri bodies, and upon progressive alterations in size and shape. The variability in structure of Negri bodies is proverbial. In size, the inclusions range from minute structures on the borderline of visibility (about 0.2 μ) to relatively large bodies 20 μ in diameter.[4] They are larger in infections produced by the "street" virus than by a virus "fixed" by frequent regular passage in the laboratory. In typical cases caused by the fixed virus, the Negri bodies may even be absent or present in such small numbers as to defy detection. A similar modification in the inclusion bodies leading to their complete absence with a change in the virus is not known in the case of any other diseases listed in this division. The inclusions may be divided into two categories:

1. The smaller ones appear to be optically hyaline. They are called "lyssa bodies" by Goodpasture (22), who believes them to be produced through a modification of the neurofibrils caused by the action of the virus. If this is proved then the material of which they are composed exists in some form in the cell prior to the action of the virus and is thus endogenous. Very little is known of the chemical constitution of the lyssa bodies except that which may be inferred from the fact that they are eosinophilic and fuchsinophilic and are easily preserved by a wide variety of fixatives. Since they exhibit so few distinctive properties their specificity cannot be considered to have been as firmly established as that of the larger more typical Negri bodies possessed of internal structure.

2. These larger Negri bodies may be vacuolated and contain the so-called "inner bodies" imbedded in a similar hyaline substance which Park and Williams speak of as the "cytoplasm" of the organism. The

[4] The limit of visibility with direct illumination is $\frac{1}{2}$ the wave length of the light used divided by the numerical aperture of the objective.

association of these two components is regarded by Negri as a prerequisite in the diagnosis of a Negri body. The inner bodies, or chromatoid elements, are markedly basophilic, yet Goodpasture claims to have demonstrated their development from mitochondria under the influence of the virus. A more likely origin would be the Nissl substance but this is merely a suggestion without evidence to support it. The chromatoid elements are subject to the greatest variation. Sometimes they are definitely grouped about the "central bodies."

"In studying the *central bodies* of these organisms, as they appear in smears, one of the first things noticeable is that they are not surrounded by a clear space —that there is no sign of a vacuolar appearance in the body as there usually is in sections. We notice next that in the great majority of the organisms the central body stands out clearly, as decidedly different in structure, and slightly so in staining qualities, from the chromatoid granules which surround it. The general type of the structure of the central body is that of many well-known protozoan nuclei; that is, the chromatin is arranged in a more or less granular ring around the periphery of the central body or nucleus, leaving an achromatic or more acid-staining center in which is situated, generally eccentrically a varying-sized karyosome. There are a number of variations from the principal type according to stage of development" (Park and Williams, 53).

The Negri bodies are, as we have intimated, very polymorphic. Some investigators are of the opinion that the changes in form and structure occur in sequence and are part of a definite life cycle. Quoting again from Park and Williams:

"All stages in transverse division seem to occur. Many evidences of budding are also seen. The chromatoid granules seem to divide and pass out with part of the cytoplasm as a bud. This budding or unequal division appears to take place very early in the growth of the organism and to continue throughout growth until the parent body forms a mass of small organisms which may then break apart at the same time. The budding accounts for the number of small and large forms in a single cell."

The reason why so much has been written on the organismal nature of the Negri bodies may be their relative inaccessibility. In spite of their large size they can only be studied with difficulty in living cells. They are best seen in the cells of the hippocampus which are embedded in a mass of fibers from which individual cells can only be isolated for examination after much manipulation and injury. Spinal ganglion cells might prove more satisfactory as material, because to isolate them is much easier. It is doubtful whether after injecting the virus into a large sensory nerve trunk they would develop large Negri bodies; but

peculiar interest attaches to the smaller lyssa bodies, because if, as seems to be the case, they are as truly pathognomonic as the other ones they will constitute a stumbling block to the advocates of the protozoan hypothesis. It will be recalled that these lyssa bodies are merely optically homogeneous droplets in which no internal organization suggestive of a protozoön can be made out.

Other evidence against the view that they are parasitic, which is recently held by Cornwall,[5] in addition to Park and Williams, is contained in a contribution by Acton and Harvey (1). These investigators have found that the "Negri bodies vary in shape, size and staining reactions in the different [kinds of] animals examined, whilst remaining constant for the same animal." One would not expect a single species of parasite to behave in this way, or to become reduced in size or disappear altogether on fixation of the virus by passage.

Molluscum Contagiosum

The molluscum bodies illustrate still another phase of the problem of specific inclusions. The evidence is stronger, that we have to do with an intracellular accumulation of living microörganisms, than in the case of any of the others and the reaction on the part of the cell to injury is less extensive. While attempts at artificial cultivation of the virus fail, the main question is whether the minute particles or "elementary corpuscles," of which the molluscum bodies are composed, present morphological and tinctorial attributes consistent with the view that they are the etiological agents of the disease?

These elementary corpuscles were first observed by Lipschütz (35) principally in smears by staining with Löffler's methylene blue and carbol-fuchsin. He described them as minute cocci and diplococci, the former about 0.25 μ in diameter, and believed them to be the etiological agents of the disease. To designate them he introduced the name, *Strongyloplasma hominis*. Lipschütz's findings seem to have been confirmed by v. Prowazek (57) who placed the diameter of the particles at less than 0.5 μ. He studied them in the fresh state by crushing cells containing molluscum bodies and found that they could be stained vitally with both neutral red and brilliant cresyl blue. For permanent preparations he fixed in alcohol and colored with Löffler's anilin fuchsin.

More recently Goodpasture and King (24) employing also a fuchsin

[5] *Indian Jour. Med. Research*, 1924–25, xii, 601.

stain have observed, (1) that these elementary corpuscles "are not derived from extruded nucleoli or from any formed cytoplasmic constituent," (2) that "the mitochondria are neither involved in their formation nor destroyed by the changes in the cell" associated with their presence, (3) that they "develop about and later within cytoplasmic vacuoles which may be regarded as the cellular response to the presence of a living foreign body."

The cytological evidence does not permit any conclusion one way or the other. The early phases in the development of the molluscum bodies appear in many respects comparable to those reported by Ludford and Findlay (42) in fowl-pox (Chapter VII) although the two diseases are otherwise quite different. They appear, it is true, to be indicative of cellular reaction to particulate foreign material such as invading microorganisms. The size of the particles (elementary corpuscles) is consistent with ready filterability, but in filtration the question of size is not alone involved. The stains employed for their demonstration are of dubious specificity especially the fuchsin combination which will color almost anything. If by this technique they were stained both blue and red like some minute Rickettsiae, it would be a strong point in favor of their being viable organisms. But it should be admitted and we must expect parasites resident in the cell and adapted to intracellular conditions to react somewhat differently from others which live outside the cell and with which we are more familiar. Nevertheless the data available would lead one to look for an organism resembling the "elementary corpuscles" which would grow in blood plasma plus skin juice at a temperature slightly below that of the body.

Infectious Myxomatosis of Rabbits

This disease deserves more attention than it has received. In addition to being highly infectious it exhibits several neoplastic attributes. Both the epidermis and the underlying connective tissue are involved. In 1909, Splendore (67) described trachoma-like bodies in the affected cells—an observation which Moses (49) was unable to confirm. De Beaurepaire-Aragão (4), on the other hand, reported the existence of intranuclear inclusions for which he suggested the name *Chlamydozoön myxomae*. During 1927, there have been two contributions to the subject.

The first, published in February by Rivers (60) afforded evidence that two types of material, represented in pink and in blue in Plate 1,

Fig. 25, are involved, the genesis of the epidermal lesions being as follows:

"Upon microscopic examination the first change noted in the epidermal cells is an increase in size. Then small, pink, granular areas appear in the cytoplasm. These areas rapidly increase in size and frequently involve most of the cytoplasm. In the center of the acidophilic masses, blue, round or rod-shaped bodies are often seen. The cytoplasmic bodies in many respects resemble the Bollinger bodies found in cells affected by the virus of contagious epithelioma of fowls. The disease process in the epidermal cells progresses until there is complete dissolution of the cells. At this time distinct vesicles appear in the epidermis, which are not unlike the ones encountered in virus diseases that usually attack the skin, *e. g.*, *herpes simplex.*"

The second contribution is by Lipschütz (40) and appeared in September. Lipschütz makes no mention of the acidophilic masses described by Rivers but devotes his entire attention to certain minute coccobacillary bodies within the cytoplasm of the histiocytes which are probably similar to those reported by Splendore as trachoma-like structures and by Rivers as blue-staining "round or oval-shaped bodies." He claims that these represent a hitherto unrecognized group of microorganisms which he calls *Sanarellia* employing, in this case, the specific designation *cuniculi*. We await impatiently further and more detailed investigations.

Lymphocystic Disease of Fish

In this condition the cellular changes are remarkable in two principal respects. The hypertrophy is enormous, for the affected connective tissue cells increase from about 5 to 600–700 μ in diameter. The internal structural changes differ from those observed under the influence of any other known virus. The specific material developed within the cells is moreover wholly different in character from others produced in this way. In the earliest stages of infection it appears in the form of spherical masses which are basophilic and later partly vacuolated. This is represented in the characteristic colors resulting from the hematoxylin and Biondi stain in Plate 1, Fig. 23. Later the inclusions become net-like and bear a striking morphological resemblance to the Golgi apparatus.

The sequence of changes following infection has been carefully studied. They are illustrated diagrammatically in Figs. 14–18. These drawings obviously cannot be made to scale because the growth of the cell is so great that to represent accurately an enlargement of a hundred or more

diameters as between cells in series in a restricted part of one page would be difficult. Briefly the alterations are as follows:

1. One or more small basophilic inclusions exhibiting a marked halo appear in the cytoplasm (Fig. 14).

2. With the hypertrophy of the cell and of the nucleus this inclusion grows large and some parts of it come to stain less intensively (Fig. 15).

FIGS. 14–18. Five cells illustrating progressive hypertrophy with the formation of a curious net-like structure in the lymphocystic disease of fish. (After Weissenberg, 1921.)

3. The lightly stained areas lose their affinity for dyes altogether so that a net-like structure results (Fig. 16).

4. The network expands, and occasionally in the same cell, younger, homogeneous inclusions may also be seen (Fig. 17).

5. The cell membrane becomes considerably thickened and the network much more complex and widely distributed (Fig. 18).

6. The contents of the cell liquefy; the membrane ruptures, and a large open cyst of irregular outline is formed. Into this cyst there is a growth of neoplastic epidermal cells.

The process is a slow one extending over several months. In its early phases it is clearly a reaction on the part of a living cell which never, however, finds expression in cell division.

Various views have been advanced concerning the origin of this peculiar network. Awerinzew (5) refers to the earliest stages of its formation as "chromidia" in support of which it can only be said that the bodies in question are basophilic. Weissenberg (75) compares them with vaccine bodies; but the material of which they are made shows no evidence of heterogeneity. According to Joseph (28) the bodies are typical cell organs "Centrophormium" (practically the equivalents of the Golgi apparatus) which are actuated by the virus and are thus to be looked upon as reaction products developed from a preformed material. The difficulty in accepting at present this interpretation is that although the networks resemble the Golgi apparatus in form their tinctorial properties are quite different. For instance they may be stained easily by the Biondi method. To settle this point one would have to ascertain the modifications, if any, in the Golgi apparatus when the first bodies appear in the cells and follow both through later stages differentially stained in the same cells. It would be of interest also to try supravital staining with neutral red in consideration of Parat's belief that the material basis of the Golgi apparatus may thus be revealed, but the thickened membrane about the cells might prevent penetration of the dye.

An intriguing problem is afforded by the extraordinary hypertrophy. The increase in diameter from say 5 μ to 500 μ involves an increase in volume of exactly a 1,000,000 times. How a connective tissue cell under the influence of the virus can add so much material to itself is difficult to understand. It is not simply a question of the taking in of water because the cell membrane also thickens, the nucleus becomes disorganized and other structural alterations take place. Whether there is a marked increase in viscosity could be determined readily by examination for displacement of the contents at different degrees of hypertrophy after centrifuging; also, by observing whether there is an increasing tendency to Brownian movement of particles in the cell. Since some lipoids, when the reaction swings to the acid side, become hydroscopic and swell, indicators might be useful; but the contents of the enlarged cells when they rupture cannot be very acid because of the

active ingrowth of epidermal cells. A study of intracellular enzymes might be interesting. Indeed, so many problems suggest themselves that space does not permit their mention.

Bearing more definitely on the subject of this chapter it is conceivable that, with such large cells, it might be possible to secure information as to the site of action of the virus concerned. The cells can easily be isolated in salt solution and in other artificial media. They are limited by an unusually stout membrane so that their surfaces could be washed with a greater hope of successfully removing adherent virus than in the case of other more fragile and much smaller cells. It might even be possible to secure some of their cytoplasm by means of a microdissection pipette in order to test its infectivity.

INTRANUCLEAR INCLUSIONS

Herpes

The intranuclear inclusions discovered in 1921 by Lipschütz (37) and by Luger and Lauda (43) occur in all forms of herpes both in man and in experimentally infected animals. Reference to Plate 1, Figs. 18 and 19, in which the inclusions are illustrated in red, will suffice to show their characteristic features.

They stain with "acid" dyes such as eosin, erythrosin, and acid fuchsin. They are larger and more irregular in shape than the plasmosomes (acidophilic nucleoli) which are occasionally met with especially in nerve cells. A clear area is usually interposed between the centrally placed inclusion bodies and the nuclear membrane on which the basichromatin becomes marginated. With these criteria in mind it is a simple matter to identify the inclusions even in the Malpighian layer of the skin where the nucleoli undergo otherwise confusing changes preparatory to keratinization.

Luger and Lauda (44) recognize definite steps in the development of the inclusions which they refer to as "oxychromatic degeneration." These are represented in Plate 1, Figs. 8–15.

1. A normal nucleus is depicted in Fig. 8. It contains some material which stains red.

2. There is an initial hyperchromatism (Fig. 9).

3. This is followed by the development of simple or multiple, homogeneous or granular oxychromatic (red-staining) masses in the center of the nucleus (Figs. 10 and 11).

4. The basichromatin (blue-staining) becomes marginated at the periphery of the nucleus (Figs. 12 and 13).

5. It condenses into droplets and begins to disappear. A clear space is often formed between the inclusion body and the nuclear membrane (Figs. 13 and 14).

6. The basichromatin is finally altogether lost and the nucleus is represented only by the central oxyphilic mass (Fig. 15). This is the terminal stage of the reaction and is very typical.[6]

The inclusion bodies thus formed, in spite of many attempts, have never been produced by other agencies than filterable, and as yet ultravisible, viruses; but when we learn more about the mode of action of the viruses it may be possible to influence the cells in a way which will lead to their formation.

There is a marked resemblance between these herpetic inclusions and others in Virus III disease (Plate 1, Figs. 3 and 4), varicella (Plate 1, Figs. 6 and 7), the salivary-gland disease of guinea pigs (Plate 1, Fig. 27), and in the so-called visceral disease (VonGlahn and Pappenheimer, 19). But this similarity may not indicate identity. It may be misleading and result from failure to employ suitable methods to bring to light differences actually existing. That part of the nucleus stains red (oxychromatin) and part blue (basichromatin) with such mixtures as Giemsa's stain and that these components change in position and in relative amount means almost nothing. The delicate techniques devised by zoölogists for nuclear study (see McClung, 48) have not been utilized and nothing has been done on the microchemical side although the way of advance is clearly indicated in a recent paper by Verne (73), which gives, among other things, an apparently reliable chemical test for thymonucleic acid. The technique indicated permits the quantitative and topographic study of this nuclear component in sections. From the physical point of view also there is a dearth of information. Data which might be significant could probably be secured by centrifugation and by microdissection.

On the other hand, certain intranuclear inclusions occur in virus diseases which do differ from the group above mentioned particularly in the behavior of the basichromatin. These will be discussed later (pp. 137 and 138).

[6] An uncertain parallel may exist for some of the alterations taking place in the formation of herpetic inclusions in the phenomenon of chromatolysis of nerve cells following injury of different kinds. A similar initial hyperchromatism occurs. Basophilic material becomes marginated leaving a central area which tends to stain with "acid" dyes such as eosin. In the disintegration of the Golgi apparatus, also, the change first occurs in the central region and the last formed material to disappear is peripherally situated. Furthermore, in the nuclear degeneration of ovarian granulosa cells a process called "chromatolysis by margination" has been described (Special cytology, New York, Hoeber, 1928, 1130).

Evidently the available material for the building up of nuclear inclusions is restricted both in amount and in variety as compared with that existing in the cytoplasm. Carbohydrates and fats are rarely present under normal conditions in the nucleus or completely lacking. The nuclei of many different kinds of cells develop the inclusions under the influence of the virus and hence must contain the formative substance, or one or more of its precursors, notwithstanding the fact that they may look quite dissimilar when viewed with the microscope. Goodpasture (21) finds that the substance of which the inclusions are built is precipitated from the nucleoplasm by fixation in Helly's fluid and may then be easily stained. Apparently, therefore, these inclusions, like the vaccine bodies, are formed by an overproduction of some substance or substances normally present in the cell.[7] This may be accompanied by little or no change in volume or by marked hypertrophy as in the lutein cells described by Goodpasture (21).

There may be still another reason, in addition to paucity of available material, why the herpetic and other intranuclear inclusions are less diversified than those arising in the cytoplasm. The difference may be dependent upon the relative isolation of the nucleus. Because of its sheltered position and limited physiological rôle, it is, without doubt, able to respond in fewer ways to environmental changes than the cytoplasm, so that one would expect reaction products to be called forth by fewer adaptive mechanisms.

As to the way in which the herpetic virus acts upon the cell, very little is known. It may enter the cytoplasm and the nucleus or it may influence directly only the surface of the cell. During the development of the nuclear inclusions changes occur in the cytoplasm which, as far as we can tell microscopically, are not specific (Cowdry and Nicholson, 15), but which nevertheless point to profound injury. The cells are killed slowly. Appearances like that represented in Plate 1, Fig. 19, illustrating intranuclear inclusions in a multinucleated cell, suggest: either, that the nuclei are living and able to multiply by amitotic division after they have developed specific inclusions, or else that the inclusions are formed after such division, most probably the former. According to Goodpasture (23) the injured nuclei can develop, within their interior, masses of myelin-like substance.

That there may be changes in the hydrogen ion concentration is

[7] A remarkable case in which wholly new material appears in the nucleus as a result of infection is that of the polyhedral diseases discussed by Glaser in Chapter VIII.

indicated indirectly by the observation of Pischinger (55) that the coloration of chromatin with toluidine blue diminishes progressively as the pH decreases and is entirely lost when the isoelectric point is reached.

Varicella

The intranuclear inclusions as they occur in varicella are represented in Plate 1, Figs. 5, 6, and 7. The similarity to those present in herpes is clearly apparent. They were first described by Tyzzer (69) in 1906. Subsequent investigation has abundantly confirmed his work regarding the inclusions but has added little to the main problem except by the successful transmission of the disease to experimental animals (Rivers, 1926) which opens up the road for further work.

Virus III Infection of Rabbits

In this condition the intranuclear inclusions look very much like those occurring in herpes but there is always the possibility already intimated, that further work may reveal slight differences. They are illustrated in Plate 1, Figs. 3 and 4. For a more detailed account than space here allows see Rivers and Tillett (61), and Rivers (59).

Salivary-Gland Disease of Guinea Pigs

The intranuclear inclusions discovered by Jackson (26) in 1920 and studied by Goodpasture and Talbot (25) and by Cole and Kuttner (11) are restricted to the duct cells. In young guinea pigs, they are indistinguishable from the inclusions already referred to in herpes, Virus III infection, and varicella, but in older ones in which the cytoplasmic and nuclear hypertrophy is very marked slight differences may be distinguished. Examination of Plate 1, Fig. 27, will illustrate this enlargement. Cole and Kuttner find that, in such cells, the inclusions themselves usually stain more intensely than typical herpetic inclusions. They describe radiating bands of basophilic material passing from the inclusion to the nuclear membrane and the occasional occurrence of a very faint network between the strands. These intranuclear inclusions are the largest known to be produced by filterable viruses, and on this account may be susceptible of study by microdissection.

Intranuclear Inclusions in "Visceral Disease"

The conditions in which these have been encountered are reviewed and discussed by VonGlahn and Pappenheimer (19) who present an additional adult case of their own. The reference is here given merely

because the intranuclear inclusions are so like those mentioned above as to indicate the probable operation of filterable viruses which are in all likelihood of more common occurrence, often in the absence of definite clinical symptoms, than has hitherto been suspected. For the present, however, each condition must be carefully considered individually on its own merits, for the reason stated, that the intranuclear inclusions called forth by entirely different viruses often appear to be indistinguishable.

Borna Disease of Horses

The intranuclear inclusions in Borna disease were first described by Joest (27) in 1911. They are strongly fuchsinophilic (*i. e.* acidophilic) and are most commonly found in the cells of the hippocampus. Judging from Joest's figures, and some of my own preparations, they are slightly different from the herpetic inclusions and others like them. They seem to occupy a relatively much smaller part of the nucleus and during their production the basophilic nuclear material remains almost undisturbed and does not marginate. Except for the presence of the inclusions, the cells are but little altered as far as one can tell by microscopic examination.

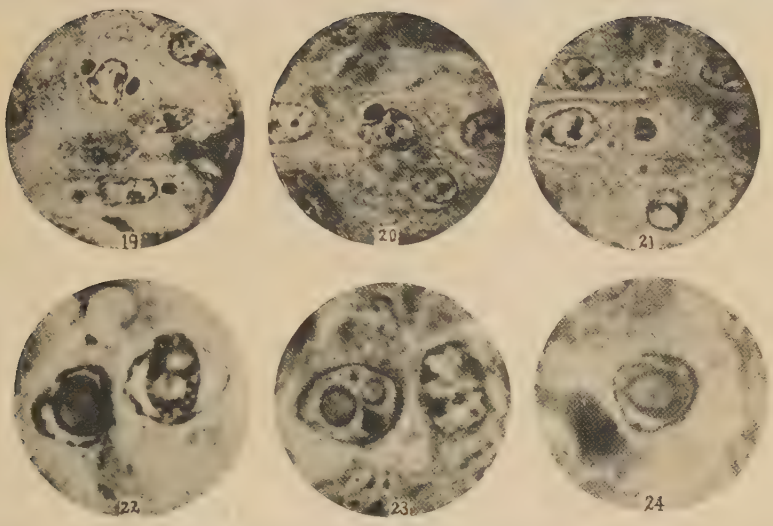
INTRACYTOPLASMIC AND INTRANUCLEAR INCLUSIONS

Smallpox

(*Cytoryctes variolae* Guarnieri, *Caryoryctes cytoryctoides* Calkins, Guarnieri bodies)

Photomicrographs of the cytoplasmic inclusions of smallpox are illustrated in Figs. 19–21. They appear as very deeply tinted bodies of round or oval shape, usually surrounded by a halo of relatively unstained cytoplasm, placed in close association with the nuclei of the affected epidermal cells. So closely do they resemble the vaccine bodies that the term "Guarnieri bodies" is often applied to both without distinction, as it was originally. To describe them in detail would therefore necessitate much repetition. The literature is given by Councilman, Magrath, and Brinckerhoff (12), by Calkins (10), and by Gins (18).

The intranuclear inclusions are likewise represented in Figs. 22–24 as they appear in photomicrographs of stained preparations. They are large spherical strongly eosinophilic structures and present many points of correspondence with the intranuclear inclusions of paravaccinia and herpes.



FIGS. 19–21. Three photomicrographs illustrating cytoplasmic inclusions in variola. (After Councilman, McGrath, and Brinckerhoff, 1904.)

FIGS. 22–24. Three photomicrographs representing intranuclear inclusions in the same disease. (After Councilman, McGrath, and Brinckerhoff, 1904.)

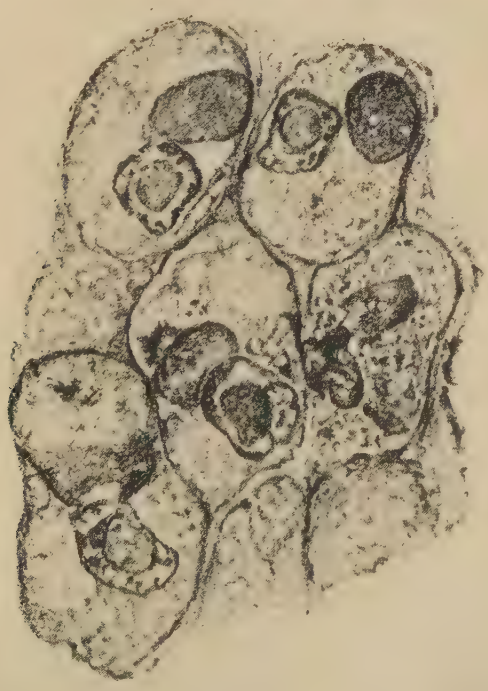


FIG. 25. Cytoplasmic and nuclear inclusions in paravaccinia. (After Lipschütz, 1919–20.)

Paravaccinia

(*Strongyloplasma paravaccinae* Lipschütz)

The specific inclusions are closely related to those of vaccinia and smallpox. The cytoplasmic ones are referred to under the same designation of "Guarnieri bodies" by Lipschütz (36). The nuclear inclusions are, like those of smallpox, quite different in character from those resident in the cytoplasm and are acidophilic and devoid of noticeable internal architecture (Fig. 25).

It is curious this ability of the viruses of variola and paravaccinia to produce in the cytoplasm Guarnieri-like bodies and in the nucleus structures resembling the inclusions of herpes, Virus III disease, and varicella. Evidently different viruses leave the same microscopically visible traces of their action although the immunity reactions are quite different. The vaccine virus may be variola virus which has been so modified that it has lost its ability to bring about a nuclear response. The change from street rabies virus to fixed rabies virus, which we can produce at will by animal passage, is accompanied by diminishing power to create inclusion bodies in the cytoplasm. To relate modifications in the viruses to alterations in cellular reaction, to search for common factors in viruses which, though different, yield inclusions which are similar, is a difficult yet fascinating line of analysis.

INCLUSIONS OF DOUBTFUL NATURE

The majority of these occur in the skin (1–4), but some are found in the nervous system (6–8), in the blood (9–10), and one in the kidneys (12).

Warts

Our knowledge of the inclusions which are found in warts, mucous papillomata, and psoriasis vulgaris is in the making. At present, the accounts are rather confusing and little can be said with assurance. In 1913, Sanfelice described intranuclear inclusions in the warts of frogs. The small and middle-sized ones took a more or less red stain by Mann's method and the large ones were colored deep blue. In other words, there was a gradation from an acidophilic structure to a basophilic one.

Two years later, Sangiorgi (64) found in human warts (verruca porro) acidophilic cytoplasmic inclusions of variable size but with a diameter sometimes attaining 7 μ. He also referred to the blue-staining of the "nuclear corpuscles." Finally Lipschütz (39) reports nuclear

inclusions in verruca vulgaris and in condyloma acuminatum which, instead of being acidophilic like those of herpes, are basophilic.

Mucous Papillomata

Ullmann (72) has found that a laryngeal papilloma from a child may be transferred to the human skin with the production of both flat warts and papillomata, and to the dog's vagina with the formation of a papilloma and further that the disease is caused by a filterable virus as is the case in warts. On histological examination, he observed acidophilic nuclear inclusions like those of herpes, and within some of these inclusions basophilic masses.

Lipschütz has discovered in experimental vaginal papillomata nuclear inclusions which, from the illustrations, look very much like those of herpes especially in consideration of the destruction of the nucleoli, the appearance of an uncolored space between the inclusion bodies and the nuclear membranes. Owing to the presence also of cytoplasmic inclusions, he believes the disease to belong in the same category with variola and paravaccinia.

Psoriasis Vulgaris

Lipschütz (38) describes an interesting variation in the staining of the nuclei of the cells of the deepest layer of the stratum germinativum. Their entire substance colors with basic dyes much more intensely than that of their neighbors.[8] He calls the modified material "chromophane" nuclear substance. There is no alteration in topographic relations within the nucleus and the changed material is not separated from the nuclear membrane by a clear space comparable to that surrounding the specific acidophilic nuclear inclusions already discussed. In his judgement, this modification in staining reaction is sufficient to place psoriasis vulgaris in the same class with variola, bird-pox, etc.

Carp-Pox

This condition has received but little attention. The inclusions, both intracytoplasmic and intranuclear, are described and figured by Loewenthal (41). They exhibit a certain similarity to those occurring in vaccinia, variola and other diseases belonging to this same ill-defined category. Loewenthal is inclined to believe that they are specific degenerations.

[8] This hyperchromatism is not unlike the alteration in staining reaction exhibited by "chromophile" cells of the nervous system (Cowdry, 13) except in the latter case the affinity of the cytoplasm for stains is also increased.

Swine Fever (Hog Cholera)

Structures said to be like the trachoma bodies have been described by Uhlenhuth and Böing (71) and by Uhlenhuth (70) within the epithelial cells of the conjunctiva. They are composed of two kinds of substances staining respectively red and blue by Giemsa's method. In 3 out of 100 cases, Uhlenhuth failed to find them in swine fever. He considers them to be cell reaction products.

Epidemic Encephalitis

The "minute bodies" discovered by Da Fano and Ingleby[9] have proved rather puzzling. They were found in the cytoplasm of cells like lymphocytes and small neuroglial cells and also extracellularly. They stain intensely with basic dyes and in the illustrations seem to be surrounded by halos. In herpetic encephalitis, Da Fano has observed similar bodies which he terms "herpetic minute bodies." He concludes that these are the virus itself "or particles of organic matter to which the virus tenaciously clings." Nicholson and I have been able in our own experiments (15) to identify the herpetic minute bodies and there is no question that in Giemsa preparations they have a suggestive organismal appearance. On bleaching the preparations, however, it was found that they are merely small particles of brownish pigment which resist the solvent action of strong hydrochloric acid. It is probable that the minute bodies in epidemic encephalitis are of the same nature but this cannot be asserted definitely until their solubility has been tested.

Nervous Distemper of Dogs

The subject is complicated because the degree of involvement of the nervous system is variable. At least three types of inclusions have been noted.

The first was described by Lentz in 1907 and clearly illustrated by him later (32). The bodies are smaller than red blood cells, stain red by his method, present no internal structure comparable to that of typical Negri bodies; but like them, are very abundant in the hippocampus. They are accompanied by a more pronounced cellular degeneration than the Negri bodies. In common with the Negri bodies, they occur in the vast majority of instances within nerve cells, but

[9] *Jour. Path. and Bact.*, 1924, xxvii, 349.

occasionally are detected extracellularly.[10] Similar bodies were reported about the same time by Standfuss (68) and their existence has been confirmed by Babes and Starcovici (6).

These authors refer to a second type, reported by Sinigaglia, which differs from that of Lentz by the fact that it presents definite internal structure. It is made up of innumerable small vacuoles containing bodies colored blue by Romanowsky staining. These were found by Babes and Starcovici not only in the brain but also in the bronchial and alveolar epithelial cells.

The third kind of inclusion is that observed by Kantorowicz and Lewy (29). It is of entirely different character consisting of a cyst-like structure enclosing many minute bodies. This parasite-like formation may, in the opinion of Goldberg and Volgenau (20), represent merely an advanced stage of chromatolysis. Findlay and Ludford (17) suggest that they may be masses of encephalitozoa. It is true that the perivascular and focal lesions as described by Roman and Lapp (62), as well as by Goldberg and Volgenau, resemble those of spontaneous encephalitis in the rabbit, but the lesions are not very specific in appearance and may conceivably be produced by a variety of agencies.

Fowl Plague

In 1905, Kleine (30) reported inclusions not unlike Negri bodies both within the ganglion cells of the brain and extracellularly. The virus is filterable and according to him the disease clinically resembles lyssa. A few months later, Schiffmann (65) examined the structures more closely and found them to have an average diameter of 15–20 μ. They are composed of an optically homogeneous or hyaline ground substance in which small basophilic "inner" bodies are embedded. They are absent in normal animals. Schiffmann notes also the resemblance to Negri bodies but does not commit himself as to the nature of the structures. Good illustrations of these inclusions have never been published and nothing seems to have been done in the past 20 years.

A Disease of Fowls in Nigeria and Palestine

Special interest attaches to the leucocytic inclusions and to the Kurloff and Todd bodies, because these conditions may afford exceptions

[10] It would be difficult to distinguish these inclusions from the lyssa bodies of rabies (p. 123) and indeed when they occur it is possible that we may be dealing with a rabic virus which has become "fixed" by frequent transfer.

to the general rule that viruses do not primarily attack blood cells calling forth specific reaction products in them. The inclusions in this disease of fowls have been described by Macfie (46) and Adler (2). They are apparently specific and definitely related to the course of the infection. On account of the fact that they are colored by Giemsa's stain even more intensely than the nuclei of malarial parasites they are called "chromatic." Adler states that:

"All varieties of leucocytes except eosinophils and mast-cells contained the above mentioned inclusions, as many as 18 per cent of the total leucocytes being infected. The nuclei of highly infected cells particularly of lymphocytes, tended to become degenerated and stained feebly with Giemsa in marked contrast to the brilliant staining of the inclusions. In highly infected lymphocytes the nucleus disappeared almost entirely and the cell stuffed with chromatic inclusions had a superficial resemblance to a Koch's blue body; this form was found particularly in tissue smears. All stages between a slightly infected lymphocyte and the forms resembling Koch's blue bodies were found in tissue smears."

Both authors are inclined to believe "that the inclusions are true parasites belonging probably to the chlamydozoa and are causally related to the disease," although there is no evidence that they are heterogeneous in terms of the chlamydozoal hypothesis and they have not been compared closely with other typical inclusions of virus diseases.

Kurloff Bodies in Guinea Pigs

Although these structures, which are found in mononuclear and lymphocyte-like cells, have been the subject of much study their nature remains completely obscure. The most recent paper dealing with them is that of Bender (7) published in 1924. She shows that they may be supravitally stained, almost specifically, with New Methylene Blue GG and that they appear in the still living cells to be homogeneous and are not included in vacuoles. These observations I am able to confirm by the similar use of brilliant cresyl blue 2 B. I have never observed an inclusion which undergoes so rapid change under conditions which, for example, will allow the study for hours of living leucocytes. After 2 or 3 minutes examination in normal saline the homogeneous appearance of the Kurloff bodies is lost and they undergo a metamorphosis every stage of which can be watched leading to the formation in their interior of bacterium-like structures colored a deep purple by the brilliant cresyl blue. Bender describes the sudden appearance of Kurloff bodies in a large stock of guinea pigs and the coincident onset of eosinophilia. There are, moreover, other ill-defined reasons, such as varying incidence

in different localities, which suggest that we may have to do with an infective process which is not manifested by distinctive clinical symptoms.

Todd Bodies

Reference is merely made to these structures, which occur in the cytoplasm of the erythrocytes of certain amphibia, to make this summary of doubtful inclusions more complete. Their appearance is illustrated in colors by Dutton, Todd, and Tobey (16) in Plate xxix, Figures 95–99. They are of two kinds: one crystalline and the other amorphous and eosinophilic. We have no inkling as to their significance and their presence has not as yet been linked with disease. The few papers dealing with them are discussed by v. Prowazek (58).

South African Horse Sickness

Kuhn (31) has found in smear preparations of the kidney, from about 80 per cent of the cases, cell inclusions stainable by the Lentz method. They vary from minute structures to bodies larger than the nuclei. Most of them are spherical and the larger ones are frequently vacuolated. They are situated close to the nuclei which they sometimes indent. Similar inclusions were not observed in normal animals. The nature of the inclusions is now being actively investigated by de Kock at the Government Laboratories near Pretoria.

DISCUSSION

An attempt may now be made to answer the questions formulated at the beginning of this chapter (page 116); but in doing so no generalization is intended.

1. We do not know even in a single case with any degree of precision the chemical nature of the material of which the inclusion bodies are built. It may be characterized negatively by saying that it is not a carbohydrate, a neutral fat, or a crystalline substance (arbitrarily understood in this connection only as one which exhibits plane surfaces and angles microscopically visible).[11] The vaccine bodies, for instance, are soluble in dilute acetic acid indicating an albuminous nature. They do not give a positive Millon's reaction, oxydase reaction, or Bensley-Macallum test for masked iron. Neither are they characteristically

[11] The polyhedral bodies of insects are crystalline and may be collected, washed and analyzed with a high degree of accuracy (Chapter VIII).

blackened by osmic acid or stained by Sudan III. During their evolution the inclusions in fowl-pox become lipoidal and blacken. Some inclusions stain red with eosin and other "acid" dyes and others blue with methylene blue, toluidine blue and other stains, but this means nothing from the microchemical point of view. Such diverse substances as the granules of mast cells, mucus, keratohyalin, and the Nissl bodies are all colored by "basic" dyes. That certain particles may be stained by a mordanting mixture like carbol or anilin fuchsin means still less. The composition of the intranuclear inclusions is more restricted than the cytoplasmic ones but here also investigators have contented themselves with data concerning their red- or blue-staining propensities. The reactions for masked iron and for thymonucleic acid (Verne, 73) which would yield significant results have not been applied. Nothing has been done with indicators and but little with the polariscope. In other words, through failure to employ available methods and to devise new ones, our information relative to the composition of the inclusions remains negligible.

2. In several cases, traces of the material giving rise to the inclusions exist in the cell prior to the action of the virus. This seems to hold for the nuclear inclusions of herpes, the vaccine bodies, the lyssa bodies of rabies and others. We cannot say, however, that all of the material comprising these inclusions is normally present in the cells; because, as the inclusions, both nuclear and cytoplasmic, grow larger, their composition changes. Obviously there may be added to the substance existing before the cell comes under the influence of the virus, other materials which arise as a result of the altered activity of the cell, or which, though produced normally in inappreciable quantities are now present in visible amounts, or which are taken in from without.

Other specific inclusions are probably chiefly of endogenous origin. Among them, those of molluscum contagiosum, contagious epithelioma (fowl-pox), and the lymphocystic disease of fish may be mentioned. To the best of our knowledge, none of the inclusions are formed through the ingestion of outside substance in the absence of a structural cellular response.

3. The relation between the developing inclusions and other recognizable cellular constituents is hard to define. When, in the beginning, we are dealing with a substance normally present in the cell, it is not simply a question of explaining a progressive elaboration of more and more of the same substance for, as we have indicated, there is reason to believe that its composition also changes. When we speak of "elabora-

tion" of material, it does not necessarily mean that the substance, whatever it is, is produced in larger amounts or more rapidly than under normal conditions. Its formation may continue unmodified and the heaping up may be a retention phenomenon due to decreased elimination or failure to utilize the material in processes taking place normally. Epidermal cells have no contiguous blood channels to carry off waste products. This alternative is not given as an intrepretation but is merely mentioned as a possibility to be considered.

The mitochondria are rarely directly involved in the differentiation of the inclusions, in the sense of actual transformation, and, to the best of our knowledge, the Golgi apparatus, never. In most instances, it is the ground substance that is concerned. Some of the cytoplasmic inclusions seem to hug the nucleus and others not. In nerve cells, the inclusions apparently arise in positions quite irrespective of the well-developed dynamic polarization. The virus inclusions in fowl-pox are first seen in the proximal cytoplasm (Ludford and Findlay, 42) in which attribute they behave like non-specific deposits such as, for example, globules of neutral fat in the pancreas. Evidently the ground substance is generally equipotential or nearly so in its capacity to elaborate the inclusions.

4. We know very little of the changes in cellular activity produced by the viruses. Unhappily the inclusion bodies have monopolized our attention. Some of them may be true microörganisms, or chlamydozoa, but the majority seem to be reaction products. They indicate the action of viruses but they have not thus far been made to tell us what that action may be. The really significant alterations in cellular activity probably precede their formation and do not reveal themselves by noticeable structural changes. The activity of the cell is shaped through these alterations in such a way that the inclusion bodies result. It is perhaps by the study of these primary modifications that clues will be obtained to the nature of the viruses themselves.

Cells may be influenced in many ways. Their irritability may be heightened or depressed. There is evidence of the former, for example, in rabies and it may occur quite commonly in other tissues which do not reveal it in such a striking manner. The questions which press for answer are whether the viruses call forth an initial increase or decrease in cellular respiration, assimilation, excretion, permeability, hydrogen ion concentration, viscosity, and in various other properties. Useful methods of investigation are at hand, especially those of pure line tissue culture.

The alterations once started and examined in their later phases are highly variable depending upon the type of cells involved and the kind of virus acting. A difference in cellular response does not necessarily signify the operation of a different stimulus. Different species exhibit distinctive Negri bodies yet the virus of rabies is the same. So also cells specialized to serve different functions in a single individual react differently to one and the same excitant. Electrical stimulation calls forth secretion in gland cells, contraction in muscle cells, and discharge of nervous impulses in nerve cells, while in the case of epidermal cells there is but little detectable response. Viruses which affect epidermal cells and nerve cells are often different but their difference should not be measured in terms of the divergence between the reaction products. The cytoplasmic inclusions are more varied than those of the nucleus for reasons already outlined.

The cells of the epidermis, which are of all the most prone to injury by these agents, are peculiar in several respects. A distinctive feature of the epidermis (and naturally of the cornea also) is its avascularity. Only one other tissue of the body resembles it in this respect, namely cartilage. Epidermal cells are relatively removed from the unifying influence of the circulation. Even the youngest cells, multiplying rapidly, are on the road to death and desquamation. The duration of their life cycle is not known. Products of their activity tend to be retained, not washed away in the blood stream. They are, moreover, subjected to forces not impinging in the same way upon other cells of the body. Thus, they are under the direct influence of definitely lowered and variable temperature, of variable light, and of a variety of mechanical injuries. What they may elaborate or break down in their interior is eliminated chiefly by desquamation, although immune bodies and certain other substances enter the circulation freely. Epidermal cells, taken by and large, except those of the very deepest layer, are, therefore, moribund reservoirs which combine an unusual isolation with the reception of many potentially formative stimuli.

Speaking generally, the cells influenced by the viruses are not immediately killed but react for hours or months to the injury strictly in accordance with their modes of life and the kind of injury. The alterations are slowly necrobiotic rather than immediately autolytic and often do not occur in neighboring cells simultaneously. Nerve cells do not proliferate but epidermal cells often multiply even after the appearance within them of typical inclusions. Epidermal cells likewise provided with inclusions frequently phagocytize and digest other cells

(vaccinia). The volume of the affected cells may remain approximately the same (rabies) or it may increase a million fold (lymphocystic disease). There is no evidence that a cell once infected ever recovers but it may conceivably do so. The formation of the inclusions may be paralleled by little visible injury to the cell or by profound injury. Eventually the cells undergo solution (cytolysis) and there may be marked leucocytic infiltration. The cytolytic phenomena resulting from the action of filterable viruses are stressed elsewhere in this book. Both may be perfectly normal processes in man. It is interesting that the extensive and rapid cytolysis in regressing mammary glands and in degenerating granulosa lutein cells are regulated by hormones which like some viruses are chemical agents of undetermined character. Leucocytic infiltration is an essential and normal step in the estrous cycle.[12] Hormones may be cytokinetic as well as cytolytic.

5. Whether the virus acts on the surface of the cell or penetrates into its interior, we do not know. We have to explain both the injury to the cell and the multiplication, or elaboration of virus. One may be a function of the other or again it may not. It is clear that a modification of the surface film of a cell might alter its whole mode of life and eventually kill it, and, further, that microörganisms can multiply in close proximity to cells without entering them. It is, however, not certain that the viruses are living agents.

The microscopically visible inclusion bodies in the diseases already discussed almost certainly are not in themselves living microörganisms; but as cellular components they undoubtedly contribute to the vital manifestations of the cells containing them though these activities may be strangely perverted. There is indeed no element of the cell which can be regarded as so inert that it does not play some part in the vital phenomena of the whole. Most of the evidence which has been cited tending to show that the inclusion bodies are independent microorganisms is open to criticism. Some of it has already been considered.

Forms suggestive of ameboid movement are usually susceptible of some other interpretation. When observed in fixed tissues it is evident that they may result from unequal shrinkage owing to their own heterogeneity or that of the surrounding medium. When studied directly in living cells the question always arises as to whether any movement seen, is their own, or that conferred upon them by mass movements of the cytoplasm. Particularly is this the case in plant cells possessed of

[12] Special cytology, New York, Hoeber, 1928, 1138, 1169, 1196.

large central vacuolar spaces surrounded by rapidly streaming cytoplasm. In animal cells when the ground substance is relatively clear movements in it are only revealed by resultant changes in position of embedded granules. The movements are more sluggish and tend to be localized, which would foster the illusion that a really passive material is moving of its own accord. The mere act of isolating a living cell, placing it in a foreign medium, and covering it with a coverglass induces an entirely new series of mechanical strains which might well necessitate changes in the position of visible elements from the places which they occupied when the cell was part of a living organized tissue. If the pressure from the slide or coverglass, or from contiguous cells on different parts of the cell is unequal it is conceivable that, in the adjustment, elements of a certain kind might move while others would remain stationary. Purposeful imitations of ameboid movement in artificial systems, while they leave much to be desired yet resemble it superficially so closely that one would hesitate to conclude that because certain elements in the cell may move that they are therefore alive.

The so-called "division figures" of the inclusion bodies rest on an equally insecure basis. When reliance is placed solely in fixed preparations, some of them are even more likely to represent fusions of material of like character than separations; for we know that in several cases the masses do fuse to form larger and larger aggregates. A good deal depends upon the position in the cell which the "division figures" occupy. Several of the illustrations in the literature, said to depict the separation of an inclusion body into two parts, suggest the existence of pressure exerted at the surface of plastic material at the site of constriction by more resilient cellular constituents such as the nucleus or cell membrane. Another possibility, especially in the streaming protoplasm of plant cells, is that a part of the inclusion body may be arrested and the rest of it drawn out by the force of the stream. If, during cell division, the inclusion bodies occur in that part of the cytoplasm where the constriction appears it is natural that they should divide passively by reason of alterations in their environment, part going to each daughter cell. Plastids, mitochondria, chromosomes, and many other cellular components divide yet no one would say that the division in any sense indicates that they are independent living microörganisms.

If the viruses are not living entities, and through cellular activity more and more of the substance identical with that which initiates the reaction is produced, it seems just a shade more likely that the material in question penetrates. Cytoplasm, specialized as it is in so many

ways, is a more complex system with larger formative potentialities than the so-called "milieu interieur" of Claude Bernard which bathes the cells although each is associated with the other through the medium of the plasma membrane (surface film). Certainly the manufacture of many known materials is commenced within the cell. This is sometimes fully accomplished before liberation and in other instances the process is apparently only completed (or the material activated) after it has diffused out through the membrane. During the increase in virus much fluid is taken into the cell and studies are needed as to its character. It may, however, be of no significance in the possible intracellular building of virus.

How the virus enters, if indeed it does enter at all, is a mystery. While it continues to be ultravisible it is difficult to picture penetration by virtue of any kind of mechanical force like motility. The cellular reaction in fowl-pox and molluscum contagiosum is suggestive of the ingestion of particulate matter but these two cases are exceptional and must be set aside because none of the viruses have been found to be particulate when viewed with the most modern dark-field and ultramicroscopic equipment, although, in a physical sense in the last analysis, they must be so.

Penetration through the nuclear membrane, as well as the plasma, or cell membrane, of viruses which produce nuclear inclusions seems on general principles to be less likely because so few outside substances in the normal course of events enter the nuclei. Their composition is rather definitely limited particularly as regards the entry of carbohydrates and fats. Their resistance to the penetration of living microorganisms is likewise quite pronounced. The Rickettsiae of Rocky Mountain spotted fever have an intranuclear phase[13] and some coccidia also,[14] but both are easily identified and wholly different in their properties from the intranuclear inclusions in virus diseases. Evidently the possibilities of surface action of the viruses on either or both membranes must be borne in mind.

The logical method of advance is to experimentally test the infectivity of materials from within the cell. An unusually favorable opportunity to do this is afforded in animals by the great enlargement of cells in the lymphocystic disease of fish already described. Their diameter may

[13] Wolbach, S. B., *Jour. Med. Research*, 1919, xli, 1.

[14] Reichenow, E., in v. Prowazek's Handbuch der pathogenen Protozoen, Leipzig, Barth, 1921, Lief. 8, 1136.

increase from 5 μ to 500 μ. But even with this advantage, microdissection technique of great delicacy would have to be employed.

In plants a more feasible method of studying the permeability of the viruses is available, namely to immerse the still larger cells of the plant, *Valonia*, in fluids containing them and then to test the contents for their presence by applying them to tissues susceptible to the viruses. It would be natural to commence with the mosaic viruses of plants (Chapter IX) and then extend the study to the viruses producing disease in animals. Microdissection would not be necessary as from 1 to 3 cc. of the sap can be obtained with ease for the infectivity tests. Exact data is rapidly accumulating concerning the phenomena of permeability in *Valonia*, thanks to Osterhout and his associates, so that information regarding the behavior of the viruses might well reveal significant points relative to their chemical and physical nature.

6. As yet none of the specific inclusion bodies has been produced by agencies other than the viruses. For this reason, they may safely be regarded as indicative of the action of disease-producing agencies of this general category. But in their identification due caution must be observed because they often differ from non-specific deposits in slight but important ways. In searching the literature, the descriptions met with are of little value as compared with good colored illustrations. Though the inclusion bodies are specific to the action of filterable viruses they are not invariably pathognomonic for individual viruses. Thus the intranuclear inclusions in herpes, Virus III disease, and varicella have much in common, and the Guarnieri bodies in vaccinia, paravaccinia, and smallpox are alike.

Because the specific inclusions have never been successfully produced experimentally, despite repeated attempts by many workers, it does not follow that they cannot be so formed. The key to the situation may be fuller information concerning the mode of action of the viruses themselves. A few of the possibilities in this connection are outlined in an earlier paragraph. If the creation of inclusions in living cells by artificial means should ever be realized, one wonders whether a virus will likewise be formed *de novo?* Until the viruses are shown to be living agents, it requires a larger stretch of the imagination to conceive of their initial development apart from living organic matter than in intimate association with it.

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EXPLANATION OF PLATE 1

Figs. 1 and 2. Epithelial cells affected by the virus of contagious epithelioma; the pink-staining masses in the cytoplasm represent Bollinger bodies. Fig. 1 also shows the result of amitotic division of the nucleus. Zenker's fixative; eosin and methylene blue stain. × 1700. From originals of T. M. Rivers.

Figs. 3 and 4. Cells, probably endothelial leucocytes, in the interstitial tissue of a rabbit's testicle affected by Virus III. The inclusions are represented by the pink intranuclear masses. Fig. 3 represents an amitotic giant cell. Zenker's fixative; eosin and methylene blue stain. × 1700. From originals of T. M. Rivers.

Figs. 5–7. Human epithelial cells injured by the virus of varicella. The pink masses in the nuclei are the characteristic inclusions. Fig. 7 represents an amitotic giant cell. Zenker's fixative; eosin and methylene blue stain. × 1700. From originals of T. M. Rivers.

Figs. 8–11. These figures represent a, b, e, and g respectively of Abb. 8 in Lauda and Luger's article on herpes simplex in *Ergebn. inn. Med. u. Kinderheilk.*, 1926, xxx, 377, and show the initial stages of oxychromatic nuclear degeneration.

Figs. 12 and 13. These figures represent a and b respectively of Abb. 9 in Lauda and Luger's article on herpes simplex (see above) and show oxychromatic nuclear degeneration fully developed.

Figs. 14 and 15. These figures represent 2 cells of Abb. 10 in Lauda and Luger's article on herpes simplex (see above) and show the terminal stages of oxychromatic nuclear degeneration.

FIGS. 16 and 17. Vaccine bodies—Guarnieri bodies—in the cytoplasm of corneal epithelial cells of a rabbit, 48 hours post inoculation. Zenker's fixative; eosin and methylene blue stain. × 1700. From originals of T. M. Rivers.

FIG. 18. Represents one cell from Abb. 18 of Lipschütz's article on the herpes group of diseases in *Arch. Dermatol. u. Syph., Orig.*, 1921, cxxxvi, 428. The cell, corneal epithelial cell of a rabbit inoculated with herpes simplex virus, shows an acidophilic nuclear inclusion. Sublimate alcohol fixation; hämalaun eosin stain; Zeiss 1/12 im. and oc. 4.

FIG. 19. Represents an amitotic giant cell in human genital herpes. From Abb. 10 in Lipschütz's article (see above). Sublimate alcohol fixation; Giemsa's stain; Zeiss im. and oc. 4.

FIG. 20. Represents a human epithelial cell affected by the virus of herpes zoster. From Abb. 1 in Lipschütz's article (see above). The characteristic change is the acidophilic mass in the nucleus. Sublimate alcohol fixation; Giemsa's stain; Zeiss im. and oc. 4.

FIG. 21. Reproduction of a cell from Fig. 5, Plate V, of Negri's article on rabies in *Z. Hyg. u. Infectionskrankh.*, 1903, xliii, 507, and shows a cell from Ammon's horn of a dog that died of rabies 15 days post infection. In the cytoplasm is a typical Negri body. Zenker's fixative; Mann's stain; Zeiss 2 mm. and oc. 6.

FIG. 22. Reproduction of a cell from Fig. 15, Plate II, of Bosc's article on sheep-pox in *Cent. Bakt., Abt. I, Orig.*, 1903, xxxiv, 413, 517, 666. It is an epithelial cell with a cytoplasmic inclusion and vacuolated nucleus. Flemming's fixative; carbolic magenta-picro-indigo-carmine stain; Zeiss 1/12 im.

FIG. 23. Represents Fig. 5 (slightly modified) of Plate xxviii illustrating Weissenberg's article on lymphocystic disease of fish in v. Prowazek's Handbuch der pathogenen Protozoen, Leipzig, Barth, 1921, Leif. 9, 1344. The cell, 27 μ in diameter, shows several cytoplasmic inclusions. Acetic alcohol fixation; hematoxylin and Biondi stain. × 1025.

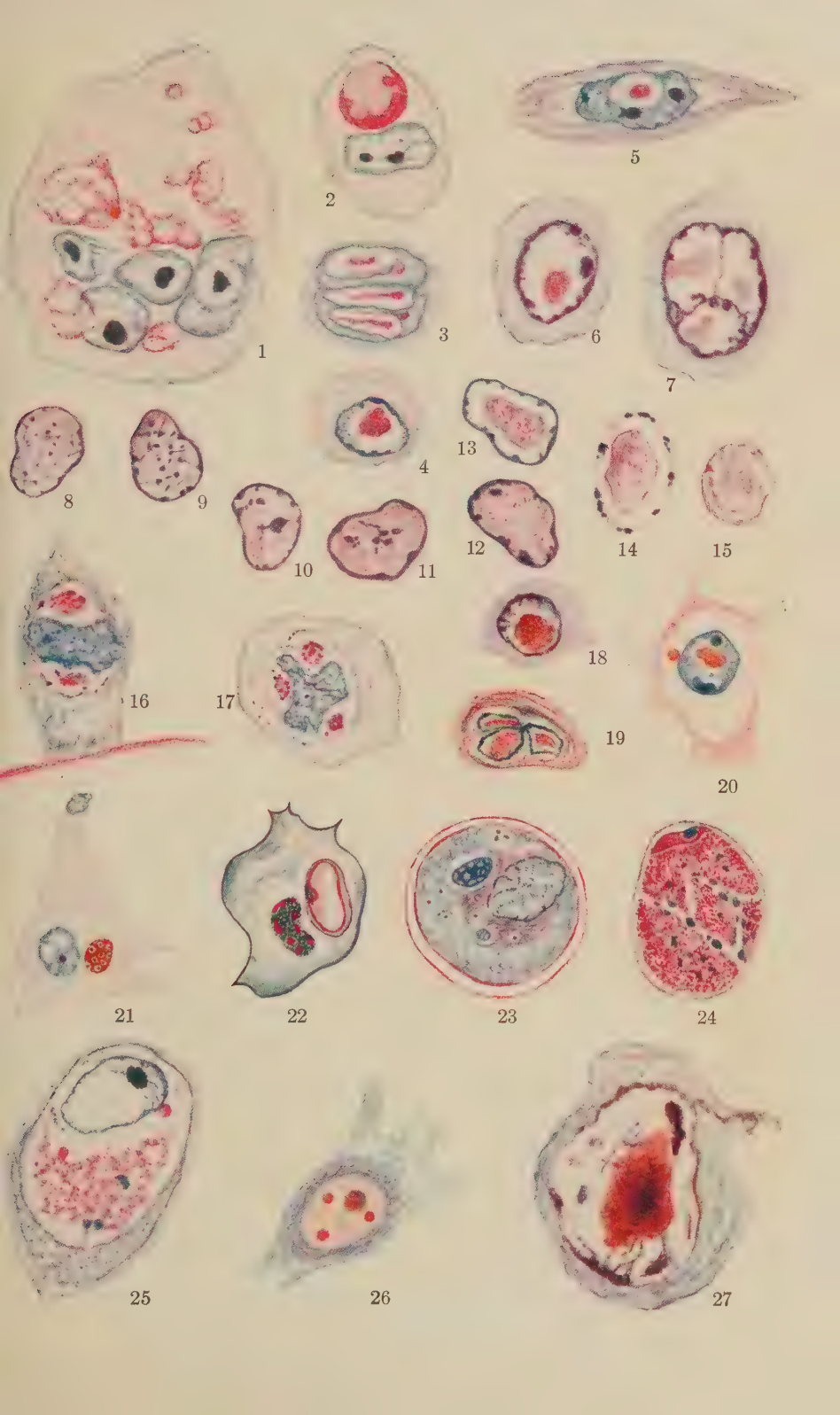
FIG. 24. Reproduction from Plate XVI illustrating Lipschütz's article on molluscum contagiosum in *Arch. Dermatol. u. Syph.*, 1911, cvii, 387, and represents an epithelial cell with its nucleus compressed and pushed to the periphery and with its cytoplasm practically replaced by a so-called molluscum body. Sublimate alcohol fixation; Giemsa's stain; Leitz im. 1/12 and oc. 8.

FIG. 25. Epithelial cell of rabbit affected by the virus of infectious myxomatosis. From original of T. M. Rivers. The cell is swollen, the nucleus is vacuolated, and the cytoplasm contains a large granular pink-staining mass in the midst of which are several blue coccoid bodies. Zenker's fixative; eosin and methylene blue stain. × 1700.

FIG. 26. Reproduction of Fig. 5, Plate II, illustrating Joest's chapter on Borna disease in Kolle and v. Wassermann's Handbuch der pathogenen Mikroorganismen, Jena, Fischer, 2nd ed., 1913, iv, 251. Ganglion cell from Ammon's horn showing pink nuclear inclusions. Lentz's stain; Zeiss im. 1/12 and oc. 3.

FIG. 27. Reproduction of one cell from Fig. 1, Plate 33, illustrating Cole and Kuttner's article on salivary-gland virus of guinea pigs in *Jour. Exper. Med.*, 1926, xliv, 855. It is a swollen duct cell with eosin-staining nuclear inclusion from a submaxillary gland of a full grown pig. Zenker's fixative; eosin and methylene blue stain. × 1700.

We owe the reproduction of this plate to the courtesy of Dr. F. B. Mallory, Editor of the American Journal of Pathology.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VIRUS DISEASES OF MAN AS EXEMPLIFIED BY POLIOMYELITIS

HAROLD L. AMOSS

CHAPTER V

VIRUS DISEASES OF MAN AS EXEMPLIFIED BY POLIOMYELITIS

HISTORICAL REVIEW

In 1840, Heine (100,101) in a monograph on paralytic conditions of the lower extremities, separated from spastic paralyses and palsies a group of flaccid paralyses the clinical picture of which he described carefully, and observed that in this group a febrile period preceded the onset of the paralysis. Previously, Underwood (241) had called attention to the fact that paralyses may follow a brief illness in children. Badham (21), in 1835, described a localized outbreak of flaccid paralyses which we now recognize as poliomyelitis. Colmer (39) described a similar outbreak in 1843. Charcot (33–35), in 1870, believed that the chief pathological process comprised a primary degeneration of the cells in the anterior horn of the spinal cord.

Strümpell (231–235), acquainted with Heine's article, published in 1884 a report on encephalitic, neuritic, and poliomyelitic types of the disease in which he saw features similar to those in Heine's cases, and suggested for the first time that the malady is infectious. His report concerning the similarity of acute encephalitis to spastic paralysis in children and to poliomyelitis was the first description of the cerebral form of the disease which we recognize today.

The first good clinical description of the disease was reported in 1890 by Medin (169–171), who studied a large number of cases in an epidemic in Sweden. The spastic type described by Strümpell was also observed by Medin. Since his report, the clinical entity became known as the Heine-Medin disease.

The non-paralytic type of poliomyelitis was first recognized by Caverly (29–31) who, in 1897, described an outbreak of 130 cases in Vermont, 6 of which had the clinical symptoms usually observed in the preparalytic stage but did not become paralyzed.

Although the infectious nature of the disease had been suggested, it was not generally accepted until Wickman (243–250) in 1905 adduced evidence of the communicability of poliomyelitis based on a study of about 1200 cases in the great Swedish epidemic. His pathological, epidemiological, and clinical studies of the acute stage of the disease are classical.

CLINICAL PICTURE

Poliomyelitis is in most instances an acute febrile disease with a more or less abrupt onset. It affects individuals of all ages, but mainly children, produces at first symptoms of a systemic infection, and later in over half the cases leads to hyperesthesias, asymmetry of reflexes, and flaccid paralyses or palsies of muscle groups.

Symptomatology

Spinal Type.—The general symptoms include fever, headache, irritability, and gastrointestinal disturbances. The febrile period is usually short, enduring from a few hours to 3 or 4 days during which the temperature is from 100° to 102.5° F. In rare instances it rises to 105°F. The febrile period may be overlooked, but in such cases the mother usually gives a history of the child's restlessness and desire for water during the night 24 hours or more before the onset of muscular weakness. Headache is generally present (95 per cent), is dull and not excruciating. Gastrointestinal disturbances (90 per cent) include anorexia, loose movements or constipation, and vomiting which occurs once or twice without projection. At various intervals of time following the onset of these symptoms the majority of cases develops flaccid paralysis of certain muscles, especially those of the extremities. Draper (44) has described these cases as the "double hump" type and suggests that the first period represents the invasion of the virus and the second period (paralysis) the localization of the virus. It is by no means certain, however, that the virus first enters the general circulation and then localizes in the central nervous system. Any of the symptoms mentioned may result from involvement of the central nervous system as they frequently occur in other infections of the brain and spinal cord. The failure to find the virus in the blood in human cases and the difficulty of infecting monkeys by intravenous injection of the active agent are opposed to the idea of general invasion with subsequent localization.

Non-Paralytic Type.—The description above applies to the usual spinal type of the disease. There are other types, of which, from the standpoint of public health, the non-paralytic group is the most important. As an epidemic progresses, the proportion of non-paralytic cases to the total number increases and may, in epidemics of considerable intensity, approximate 40 per cent. These patients have the usual symptoms of the disease with positive spinal-fluid findings, but do not become paralyzed. These cases were formerly classed as "abortive."

Perhaps it is better to restrict this term to the cases in which only the first "hump" occurs or to those with only slight unexplainable symptoms appearing during epidemics of poliomyelitis.

Spastic Type.—Spastic types of the disease occur especially in patients with encephalitic symptoms. The cell count in the spinal fluid is high. The prognosis in this type, though apparently very unfavorable, is really very favorable.

Bulbar Type.—This type is seen after an epidemic is well established. The onset may be insidious. A child, previously healthy, becomes listless, refuses to eat, and perhaps has difficulty in swallowing. The fever may be very mild, a slight headache is not unusual, and frequently there is no complaint. Nothing definite is found on examination except perhaps a slight reddening of the fauces and an excess of mucus in the throat. There may be no obvious palatal palsy at this time. The reflexes may be only mildly hyperactive without the usual asymmetry. The stiffness of the neck may not be greater than that observed in cases of simple meningismus. Such a picture presenting itself suddenly when one is not thinking of poliomyelitis does not, of course, suggest the disease. Pleocytosis of 15–100 cells per cubic millimeter in the spinal fluid is almost certain to be present, but globulin is only slightly, if at all, increased. Facial paralysis, usually asymmetrical, and ocular palsies may be the first outward signs of the true nature of the disease, and as a rule occur within a day or two after the onset of the malady.

Ascending Paralytic Type (*Landry's*).—In this type there is the usual febrile period and unilateral paralysis of the lower extremities, but the acute stage endures and then the other leg, the arms, the abdominal muscles, the intercostals, and finally the diaphragm become involved. The mortality in untreated cases is almost 100 per cent.

Physical Examination

During the febrile period, the child, if undisturbed, is usually dull and listless, but is extremely irritable when aroused. Often the patient, having learned that handling causes pain, turns away when approached by the examiner. Pain on slight pressure of the muscles and on passive motion is quite characteristic. In many cases, there is exquisite hyperesthesia of the skin (posterior root ganglia involvement). One leg may be colder than the other and there may be localized sweating presaging paralysis (sympathetic involvement). The neck is consciously unyielding as bending of the neck and back causes pain (stretching of the involved meninges). Indeed this is one of the most valuable diagnostic

signs. In attempting to elicit Kernig's sign, the back is bent and a spurious, mildly positive result is obtained.

The reflexes are first hyperactive and later abolished in the paralyzed members. Thus there is the great tendency to asymmetry, especially of the reflexes below the waist. The presence of the neck and back sign, fever, irritability, and especially asymmetry of the reflexes in a child in which there are no obvious causes for the symptoms are indications for lumbar puncture.

Spinal Fluid.—Diagnosis cannot be made by means of spinal-fluid examinations alone but the findings considered in relation to time of onset of symptoms offer valuable information, and it is possible to make a presumptive diagnosis before the onset of paralysis, especially during epidemics of poliomyelitis. Pleocytosis (1) is the rule, even early in the course of the disease, and consists of from twelve to several hundred, even 2000, (mostly lymphocytes) per cubic millimeter. Globulin (94) is only slightly increased during the first 24 hours but appears in increasing amounts until the third day when the reading may be ++, and endures longer than the pleocytosis. This is in striking contrast to the very early high globulin content in the spinal fluid from patients with meningitis and is of great assistance in differential diagnosis. Of course, the character of the cells varies: in most cases, in addition to the lymphocytes, there are bilobate cells, desquamated endothelial cells, and in many instances polymorphonuclear cells which constitute 40 per cent of the total number. The bilobate cells occur early, and within 24 hours there is a marked drop in the relative number (Draper, 44). Lyon, in a paper to be published soon, states that the replacement of bilobate cells by mononuclear cells is pathognomonic of poliomyelitis.

It is important to make the spinal-fluid count at the bedside: (1) the spinal-fluid findings should be considered as a part of the clinical picture and in relation to the other findings, (2) agglutination and cytolysis produce errors in proportion to the time elapsing between the collection and the examination of the fluid. Changes in the spinal fluid are probably due to the mild inflammatory reactions of the meninges in which lymphocytes predominate.

Blood.—It is generally accepted that abnormal white blood counts are constantly found in poliomyelitis, but opinions concerning the characteristics of these abnormalities vary.

Müller (178, 179) asserts that a distinct leukopenia with a relative lymphocytosis is pathognomonic of the febrile stage, while La Fetra (124) describes a moderate leukocytosis as being characteristic of the acute stage in human beings.

Gay and Lucas (95) summarize their blood findings as follows: "The acute stage of anterior poliomyelitis as it occurs in human beings, and as it is produced experimentally in monkeys, is characterized by the occurrence of a distinct leukopenia. The differential count shows a relative increase in number of eosinophils and lymphocytes."

Peabody, Draper, and Dochez (200) found in human cases a constant and marked leukocytosis. They also found a constant increase in polymorphonuclear cells of from 10 to 15 per cent and a diminution of lymphocytes of from 15 to 20 per cent.

H. D. Taylor (237) made blood counts on six series of monkeys before and at various intervals after inoculation with poliomyelitic virus, and used as a basis of comparison averages of 121 counts on 40 normal monkeys. He found that

"all the counts made on monkeys during the course of typical acute experimental poliomyelitis show a variation from the normal; . . . . after injection of active poliomyelitic virus the lymphocytes are diminished but return to their former number and are actually increased between the fourth and sixth days of the incubation period. The polymorphonuclear count is high at this time. The normal average of lymphocytes of 11,815 is increased to an average of 19,696 though the average percentage is slightly lowered. During the first 3 days after onset, a marked decrease in the lymphocytes takes place. Thus, instead of an average normal lymphocytic count of 11,815 the number is 3,302, and the average percentage is 15.6. At this time the polymorphonuclear neutrophilic leukocytes are materially increased in number (18,231) as compared with an average normal of 9,116, and the percentage is also increased from an average of 41 to 84 per cent of the total white cells. When the monkeys become completely prostrate, metabolism is at low ebb, and there occurs a further decrease in the actual number of lymphocytes which remains low for long periods. The total number of polymorphonuclear neutrophilic leukocytes returns to normal, but there remains a relative increase averaging 77 per cent. Finally, during recovery both types of cells return to the average normal count and relation. No stimulation of the lymphocytes above normal appears during recovery."[1]

Prognosis

The mortality in poliomyelitis is very variable ranging from 7 to 25 per cent with an average of about 14. There seems to be some relation between mortality and age of the patients. In Wickman's (245) series, the mortality in patients less than 11 years old was about 12 per cent, but between 12 and 32 was more than double (28 per cent). In a Swedish epidemic, it reached 42.3 per cent. Death usually comes on the

[1] Copied from an article by Amoss in Tice's System of medicine, Hagerstown, Md., W. F. Prior Co., 1921, v, 335.

fourth or fifth day and rarely after the eighth unless there is complicating pneumonia in cases with respiratory paralysis. The prognosis is best in patients with the encephalitic type and in those who are irritable or in coma early in the course of the disease.

Prognosis as to paralysis is likewise variable but in general is better in the younger children than in the older. Partial and even completely paralyzed muscles may recover gradually or quickly within 48 hours. Wickman analyzed 530 cases: there was paralysis in 56 per cent. The figures in Massachusetts (107) and Vermont (31) are higher and, of course, will vary in different age groups, in different epidemics, and according to the accuracy of diagnosis especially among the non-paralytic types.

PATHOLOGY

Macroscopic Pathology

The gross appearances in the uncut brain and cord of the human case and the experimental disease may not present any abnormality except a slight bulging of the mass when the dura is cut. In many cases the meninges are congested. On section the brain and cord are moist and seem to be slightly swollen. There is usually congestion of the capillaries of the grey matter, sometimes of the white substance, and here and there petechial hemorrhages. A cross-section of the cord, especially through the cervical and lumbar enlargements, brings out the chief macroscopic change. At one or both of these levels the grey matter rises slightly above the cut surface and varies from a diffuse pink with an occasional capillary hemorrhage to an obvious hemorrhagic myelitis. The lesion may be present in the lumbar enlargement and diminish in intensity in succeeding proximal sections; also the lesion may be present in the cervical and diminish in succeeding distal sections. In human cases the medulla is usually distinctly congested, although it often happens that no lesion is visible macroscopically.

Microscopic Pathology

The virus provokes a round cell reaction. There is always more or less response in the meninges, especially around the blood vessels in the pia. The reaction may be mild and localized at certain levels of the cord, or intense and generally distributed. A few polymorphonuclears and cells of local origin may be found among the lymphocytes. The meningeal reaction is probably the first lesion in the disease and is the organic basis of the neck and back sign observed clinically.

The next lesion in order of occurrence is found in the posterior root ganglia. Here the usual picture is that of focal infiltration of small lymphocytes accompanied by a midinterstitial hyperplasia and various degrees of nerve-cell destruction. The latter progresses in the same manner as in the motor cells in the cord to be described later. The posterior root ganglia are the first nerve tissues to be attacked and are also the first to recover. It is remarkable that with such extensive lesions as sometimes occur, there are no residual disturbances of sensation. The changes in the posterior root ganglia probably explain the exquisite hyperesthesia. The lesions in the Gasserian, ciliary, and sympathetic ganglia correspond to those described in posterior root ganglia.

In the substance of the cord, both the white and the grey matter are affected. The lesion in the former consists of perivascular infiltration about the capillaries, slight edema, and sometimes hemorrhage. It is the grey matter which suffers most. Much attention has been directed to the changes in the anterior horns; however, the posterior horns are nearly as often involved in the acute process.

There are focal, perivascular, and diffuse infiltrations of round cells and edema, especially around the blood vessels and the nerve cells. The blood vessels in many instances are still further damaged resulting in small or extensive hemorrhages. The blood vessels outside the central nervous system are apparently not damaged to this extent. It would seem, therefore, that the injurious effect is associated with the multiplication of the virus. The nerve cells seem to be especially susceptible either to the direct or indirect effects of the virus. Examples of the changes to be described may be seen in a single section. The sequence of the cell changes, of which the first step is the perineuritic edema, has been followed by killing monkeys at various stages in experimental poliomyelitis. Later the protoplasm of the cell becomes granular, the nucleus loses its finer markings and stains more faintly. During this time neuronophages are arranging themselves about the cell, the peripheral protoplasm seems to dissolve leaving a coarse, granular débris and coincidently the neuronophages insinuate themselves into the dead cell. After solution is complete, scar tissue fills the space previously occupied by the nerve cell.

Permanent damage to the nerve cells results in paralysis and, if by chance the respiratory centers are involved, death ensues. It is a point of especial importance that cells, damaged to the extent that they do not function, may recover as shown by the occasional rapid return to normal

of completely paralyzed muscles. This fact suggests the edema as the immediate cause of the inability of the cell to function. The pressure and isolation of the cell from fluid and gaseous interchange would be expected to affect the function of the cell and, if prolonged, lead to its death. Some attempts have been made, by intravenous injection of hypertonic solutions, to ascertain whether the nerve-cell injury is due to edema or to a toxin. But edema in this disease is apparently not a question of osmotic pressure alone. There is no positive evidence of the presence of toxins, and, since toxins are usually firmly bound and recovery of nerve tissues is slow after toxin union, it is difficult to reconcile the rapid restoration of function with the hypothesis that the damage resulted from this cause. The question must be left open with the weight of evidence in favor of the edema.

Distribution of Microscopic Lesions in the Brain and Cord.—In the majority of human cases the lesions are present in the medulla though they may be slight. The cord is more often involved than the brain, but in both the distribution is irregular. In the cord the lesions are more often present in the cervical and lumbar enlargements, while in the brain a frequent site is beneath the floor of the fourth ventricle. The basal ganglia and subcortical regions of the cerebrum are sometimes involved, and lesions may also be present in the cerebellum. At autopsy it is the custom to take blocks of tissue from the cortex, basal ganglia, medulla just above the calamus, cervical and lumbar enlargements and middorsal portion of the cord, and at least two posterior root ganglia from the cervical and from the lumbar level.

Lesions Outside the Central Nervous System

All the lymph glands, including the lymphatic tissue of the spleen are hyperplastic (252). The virus is found in the mesenteric lymph nodes. In the liver small focal infiltrations of round cells are found sometimes with small areas of necrosis. The hepatic changes in human cases vary and are sometimes found only after diligent search.

ETIOLOGY

Experimental Transmission

The infectious nature of poliomyelitis was fairly well established by the observations of Wickman who focused attention upon the acute stage of the disease and, by his epidemiological studies, demonstrated its communicability. In May, 1909, Landsteiner and Popper (133) published a report of two successful inoculations of monkeys with the

spinal cord obtained from two fatal cases of poliomyelitis. The injections were made into the peritoneal cavity. One monkey became paralyzed in the lower extremities and died on the sixth day after inoculation; the other was killed on the nineteenth day. In both, lesions of the spinal cord, similar to those in man, existed. The disease could not be transferred to other monkeys. In September of the same year, Flexner and Lewis, using the more direct route of inoculation, namely, injection into the brain, were able not only to establish the experimental disease in one monkey, but to transfer it from monkey to monkey by subdural injection of suspensions of the spinal cord and, finally, of the cortex. In their first report (73) they stated that they had been able to carry the virus through three passages. Since that time, the original M. A. virus has been passed through several hundred monkeys. These findings were confirmed by Strauss and Huntoon (230,) by Leiner and v. Wiesner (137, 138) and Landsteiner and Levaditi (126). Flexner and Lewis (74) showed that after the virus had been established in a series of monkeys it could be carried on indefinitely in these animals. They were able to produce the experimental disease by injections of virus into the sciatic nerve, the circulation, the peritoneal cavity, and the subcutaneous tissues. None of the observers was able to find bacteria or protozoa in the poliomyelitic tissues or to cultivate in any of the media then available any kind of microörganism. Flexner and Lewis (75) found that the virus was still viable after remaining in glycerol (concentration not stated) for 7 days. These workers also demonstrated that poliomyelitic cord saturated with sterile quartz sand, mixed with salt solution, thoroughly shaken, and put through a Berkefeld filter was able, when injected intracerebrally, to produce the experimental disease. They conclude, "In the foregoing experiments, taken in conjunction with negative results of bacteriologic and histologic examinations, it would appear that the infecting agent of epidemic poliomyelitis belongs to the class of minute and filterable viruses that have not thus far been demonstrated with certainty under the microscope." On January 1, 1910, they reported (76) further studies of the properties of the virus. The second strain which they isolated (K) was found to retain its virulence, apparently unimpaired, on being kept constantly frozen at $-2°$ to $-4°$C., in the Frigo apparatus for a period of at least 40 days; and also when kept for at least 50 days at a temperature of about $+4°$C. During this time the specimen of spinal cord became slowly softened through autolysis and overgrown superficially with mold. They found also that the virus was not killed by being

suspended for at least 7 days over caustic potash. The possibility that the action of the virus is due to soluble toxic bodies and not to living organisms was excluded by transferring the disease by means of the spinal cord obtained from monkeys which succumbed to a filtrate (74). They reinoculated several of the monkeys which had passed through an attack of the experimental poliomyelitis and found that in some instances, the virus which caused paralysis in the control monkeys produced no infection of the previously inoculated monkeys. They conclude that an attack of experimental poliomyelitis affords definite immunity to reinfection. They were unable to alter the course of the disease following an intracerebral inoculation of active virus by the simultaneous injection beneath the skin of a virus inactivated by heating. They were unable to confer the experimental disease upon rabbits, guinea pigs, a horse, a cow, goats, pigs, sheep, rats, mice, dogs, and cats (76).

Methods of Obtaining the Virus

Necropsy, as in a human case, should be performed as soon after death as possible. The brain and cord are removed aseptically. The operator should wear sterile rubber gloves. Practically all of the tissues of the central nervous system contain the virus, but it is apparently present in greater concentrations in the lumbar and cervical enlargements of the cord and in the basal ganglia of the brain. Small samples of these tissues are ground in a mortar with sterile quartz sand, and sufficient sterile isotonic salt solution is added to make a 10 per cent suspension. A portion of this is plated by the ordinary method for the detection of contaminations. If after incubation of this poured plate no growth is found, the procedure for the establishment of the virus in monkeys is as follows:

With sterile instruments, small pieces, weighing about a gram, are taken from the lumbar and cervical enlargements and from the basal ganglia and weighed together on a sterile watch-glass. They are then transferred to a sterile mortar, mixed with 1 cc. of sterile quartz sand, and ground until the mixture becomes a smooth paste, when a few cubic centimeters of salt solution are added. The calculated amount of isotonic salt solution (amount necessary to make a 5 per cent suspension) is added and the suspension is put into a tall glass-stoppered bottle and shaken by a machine for at least 15 minutes. The suspension is now transferred to a sterile centrifuge tube and whirled for 3 minutes at half speed. One to 2 cc. of the supernatant material is injected very slowly through a small trephined opening directly into the cerebrum of the anesthetized monkey and the remainder (about 45 cc.) of the suspension is injected intraperitoneally. If it is desirable to establish a particular strain of virus, more than one monkey should

be used. If, after 10 days, the monkey shows no signs of excitability, fatigability, and muscle weakness, reinjections into the same monkey with fresh suspensions are made. If the human tissues are found to be grossly contaminated, they are placed in 0.5 per cent phenol in isotonic salt solution and kept in the icebox for 7 or 10 days before the suspension for inoculation is prepared. To prepare the suspension the tissues are washed several times in isotonic salt solution and a suspension made as already described. After the preparation of the suspension, the remaining tissues are preserved in 50 per cent sterile glycerol in isotonic salt solution at a temperature of 4°C. The injection of subinfective doses does not confer a refractory period but apparently renders the monkeys more susceptible and the chances of establishing the experimental infection are increased.

The mortality among monkeys injected with the human virus is very low, varying from 20 to 40 per cent. It is, therefore, necessary to sacrifice the monkey on the first passage as soon as the experimental disease is well established as shown by the period of excitability, fatigability, and definite muscular weakness. The brain and cord are removed as at the human autopsy and suspensions made in the same manner for injection into monkeys for the second passage. In each succeeding passage in monkeys, the mortality mounts until practically every monkey having the experimental disease becomes prostrate and dies of respiratory paralysis. Correspondingly less and less amounts of the virus are necessary to produce the experimental disease so that finally when the mortality approaches 100 per cent only 0.001 cc. is necessary to produce the experimental disease. On repeated passage and storage in the interim in glycerol at 4°C., the virulence of the material endures, but, after several years, the activity of the virus decreases on further passages and the amount necessary to produce the experimental disease becomes larger and larger. After still further passage, however, the cycle is apparently renewed and the virus regains its infectiousness. These fluctuations in virulence may have some epidemiological bearing and have been discussed by Flexner, Clark, and Amoss (86).

Symptomatology of Experimental Poliomyelitis

There is no experimental disease which so clearly reflects in its pathological and clinical aspects the human analogue as does experimental poliomyelitis. Intracerebral or intravenous injections of the virus produce no immediate effects. The inoculated monkeys are apparently normal when they recover from the anesthetic and remain normal during the period of incubation which is from 3 to 24 days (usually between 6 and 9 days), varying with the individual monkey, the virulence of the virus, and the route and amount of injection. In the prodromal period,

the monkey becomes excitable, the fur is ruffled, there is a high-pitched staccato cry, and the monkey tires easily when allowed to run in the large cage. Within a few hours, ataxia appears, there is tremor of the arms, legs, or head, and when not disturbed the monkey retires to a corner of the cage where he sits dejectedly as though he were very ill. Within the next few hours, the ataxia merges into a definite flaccid paralysis, and a facial paralysis appears as shown by the unilateral grimacing. There is a quick extension of the paralysis to include practically all of the voluntary muscles except those of the tail, the jaws, and the diaphragm, and the monkey is prostrate. The temperature becomes subnormal, the animal gradually gets weaker and finally expires within 3 days after becoming prostrate. The pathological changes are in every way comparable to those found in human poliomyelitis. Should the animal recover by careful nursing or by the administration of immune serum, the paralyzed muscles which do not return to normal become atrophied.

Viability of the Virus

Confirming the reports of Flexner and Lewis of the resistance of the virus to glycerol, already referred to, Thomsen (239) found the virus still active in poliomyelitic tissue which had been preserved for 2 years at −15°C. in 50 per cent glycerol. He also found that a filtrate from a suspension of one part of poliomyelitic brain and cord plus five parts of isotonic salt solution was active after 25 months at 0° to 4°C. There was a definite diminution in the virulence of the virus as shown by the fact that the first suspension required only 0.003 cc. to produce the disease when injected intraperitoneally into monkeys, but after 22 months 1.0 cc. was required to obtain a similarly positive result. Autolysis of tissues at 4°C. did not kill the virus. Flexner and Amoss (65) found that the virus could be preserved for 4 to 6 years in 50 per cent glycerol, but that larger amounts of the virus must be injected to produce experimental poliomyelitis. The virus remains active in 0.5 per cent phenol for 15 months. The virus was not extremely resistant to autolysis at 37°C. as it becomes inactive after 20 to 30 days (84, 90). It remains active in sputum and nasal secretions from 6 to 7 months. The presence of colloidal substances with adsorptive power destroys the virulence after 1 or 2 days. The thermal death point is 50°C. for ½ hour. It is killed by light in the presence of eosin and by weak solutions of mercuric chloride, but is not affected by ether. Amoss and Aycock (unpublished experiments) found that the virus remained viable

for at least 48 hours at hydrogen ion concentrations varying from pH = 5 to pH = 8.2.

Filterability of the Virus

Early in the work on experimental poliomyelitis, Flexner and Lewis (74) found that the virus would pass through a Berkefeld filter (porosity not stated) and this observation was quickly confirmed by other observers.

To test the filterability of the virus, a suspension is made in the manner already described in which 5 gms. of poliomyelitic nervous tissue are ground and shaken in 100 cc. of isotonic salt solution. It is centrifuged, filtered through paper, and passed through the candle by means of a suction apparatus. The pressure filter at 30 lbs. per square inch is commonly used. The virus passes through a Berkefeld candle V (coarse) with great ease, through candle N easily, but is much reduced in amount by passing through the finer candle, W. It has been found to pass through the corresponding grades of Chamberland filters. In unpublished experiments made several years ago, we found that the virus, if given sufficient time at 100 lbs. pressure, would pass through a layer of congealed gelatin of 5 mm. thickness. The virus cannot be differentially centrifuged by the method employed by MacCallum and Oppenheimer in their work with vaccine virus. A clear watery filtrate either under the dark field or by staining methods contains no granules which can be associated with the virulence of the suspension.

Bacteriology

Globoid Body.—A great many workers have reported diverse forms of microörganisms thought to have a close etiological relation to the disease. One of the most promising was reported by Flexner and Noguchi (83, 84) who isolated the globoid bodies from poliomyelitic tissue. Somehow, the general impression is abroad that the globoid bodies are rather indefinite structures. They are exceedingly small, but very distinct, and are undoubtedly organisms.

The first isolation of the globoid body is usually accomplished with difficulty by placing bits of poliomyelitic tissue or filtrate into the Noguchi medium consisting of human ascitic fluid to which fresh sterile rabbit kidney tissue is added. The ascitic fluid is overlaid with sterile paraffine oil and a few plates under strictly anaërobic conditions in a hydrogen jar are incubated at 37°C. Any tube showing marked turbidity, coagulation, or gas production within 1 or 2 days may be set aside as grossly contaminated. After a 5-day incubation, a faint opalescence appears about the fragment of the tissue at the bottom of the tube. After

10 or 12 days, the diffuse opalescence of the medium in the tubes in which growth has been observed to take place begins to diminish as sedimentation sets in, during which minute irregular particles form, which are heavy enough to fall slowly to the bottom of the tube. Transplants may be made to fresh medium by withdrawing from 0.2 to 0.5 cc. from the bottom of the tube with a capillary pipette and placing it by means of the same pipette in the zone around the kidney in the fresh medium. After a few transplants, cultures may be obtained in a solid medium consisting of ascitic fluid and sterile rabbit tissue to which a suitable quantity of 2 per cent nutrient agar has been added. After several days incubation, a diffuse opalescence appears about the tissue fragments. This opalescence gradually changes into visible grains which are later recognized as colonies. Staining of the colonies is accomplished by fixing a smear in methyl alcohol for an hour, washing in distilled water, and then immersing the slide for 12 to 14 hours in a solution containing one drop of Giemsa's stain per cubic centimeter of distilled water. The globoid bodies are arranged in paris or short chains or in small aggregated masses of a bluish or violet color. The individual microörganisms average 0.2 to 0.3 μ in diameter. In detecting the growth in fluid medium the dark field may be used to advantage.

With each transfer the organism grows more easily and diffusely and incidentally becomes less virulent. Mass cultures (90) for immunological work (3) may be obtained by seeding ascitic fluid agar plus rabbit kidney tissue contained in a 250 cc. Erlenmeyer flask. After the agar has solidified, 100 cc. of beef infusion broth, pH = 7.4, are added and the whole is overlaid with paraffine oil. It is not necessary to place these flasks in an anaërobic jar. The organisms grow first in the solid medium and then up into the broth forming a heavy layer at the bottom of the broth without producing turbidity.

Pathogenicity of the Globoid Body.—Typical experimental poliomyelitis has been produced in monkeys by the injection of the first, fourth, fifth, eighteenth, and twentieth generations of the globoid body. If 0.5 cc. is transferred from the first tube of fresh growth to new tubes containing 12 cc. of medium and if this procedure is continued in succession to the eighteenth, the dilution at the eighteenth transfer is twenty-four to the seventeenth power which is far beyond the limits of infectivity of the original material used. Flexner, Noguchi, and Amoss (90) were able to produce typical experimental poliomyelitis by mass cultures of the globoid bodies obtained from the eighth generation on solid media which had been kept at 37°C. for 13 months. Many experiments have shown that, if no growth occurs from the first culture of poliomyelitic tissue in Noguchi medium, the virus survives 20 but not 30 days under these conditions. Noguchi (84) found the organism singly in tissues. Amoss (2) incubated poliomyelitic tissue in Noguchi medium for 12 days, poured off the ascitic fluid, and by means of a special hardening method (236) was able to prepare a section and color

it with Giemsa's stain. In these preparations he found chains of five to eight minute bodies corresponding in size and pictorial properties to the globoid body. Since the organism is found in human poliomyelitic tissue, since it can be cultivated therefrom, since injection of cultures produces experimental poliomyelitis, and since it can be cultivated again from the tissues of animals in which poliomyelitis has thus been induced, it seems that Koch's postulates have been satisfied. Thus at one time the probability that the globoid bodies play the chief etiological rôle in the production of poliomyelitis seemed clear. The globoid bodies, however, have not been found in highly infectious filtrates, nor could Amoss (3) detect any immunological relationship between the globoid bodies and the true virus. From considerable experience, we realize that the relationship of the globoid bodies to poliomyelitis remains unsolved (Flexner, Amoss). These small organisms may be unusual forms of the true virus, which is unlikely, or they may adsorb and keep viable the true virus.

Streptococci.—Since it is so easy to cultivate streptococci from poliomyelitic tissue, several workers (167, 168, 219–224) have adopted the very obvious conclusion that in this group of organisms one finds the cause of poliomyelitis. In the first place, the various strains of streptococci isolated from human and monkey poliomyelitic tissue have not been shown to belong to the same group. The relationship of streptococci to poliomyelitis may be demonstrated by the following simple experiment:

> Infective doses of the virus filtrate from which no streptococci can be cultivated are injected into two monkeys at the same time. Monkey A is sacrificed as soon as definite paralysis appears. From the brain and cord of this monkey, which can be shown to contain the virus by inoculations, cultures on every kind of medium available are made but no streptococci are found. Monkey B is allowed to become prostrate and die as a result of respiratory paralysis. Obviously this animal corresponds to the fatal human case of poliomyelitis. Streptococci are easily cultivated from the brain and cord of this monkey. These organisms may be thus considered as agonal invaders.

Bull (27) found that streptococci isolated from the poliomyelitic brain and spinal cord of monkeys which succumbed to inoculation with the filterable virus failed to induce in monkeys any paralysis or the characteristic histological changes of poliomyelitis. Monkeys which have recovered from infection with streptococci derived from cases of poliomyelitis are not protected from infection with the filtered virus and

their blood does not neutralize the filtered virus *in vitro*. Bull (27) regarded these streptococci as secondary bacterial invaders of the nervous organs and failed to detect any etiologic or pathologic relationship between streptococci and epidemic poliomyelitis in man, or true experimental poliomyelitis in the monkey. He calls attention to the fact that, in the many thousands of reported cases of poliomyelitis, no instance of metastatic infection and inflammation such as occur usually in streptococcus infections have been reported.

Routes of Injection

In addition to the intracerebral injection which requires the least amount of virus for the production of experimental poliomyelitis, various other routes may be employed which require correspondingly larger amounts in the order named: intraspinal, intrasciatic, intraocular, intranasal, intraperitoneal, subcutaneous, intravenous.

Intravenous Route.—By the intravenous route, enormous doses of the virus are necessary to produce poliomyelitis, and then, if the central nervous system is intact, the infection is inconstant. If, however, normal horse serum, monkey serum, human serum, isotonic salt solution, or Ringer's solution is injected intraspinally 12–18 hours prior to the intravenous injection of virus, infection is easily accomplished. The same is true with nasal applications of the virus for a short period. These two conditions will be discussed under pathogenesis.

Gastrointestinal Route.—Leiner and v. Wiesner (139) were able to infect monkeys by the gastrointestinal (88) route, they accomplished this by feeding large amounts of the virus to monkeys after having neutralized the hydrochloric acid of the stomach with sodium bicarbonate and reducing the motility of the intestinal tract by large doses of morphia. Two of their five monkeys came down with experimental poliomyelitis. We were unable to infect half-grown monkeys by this route following the procedure of Leiner and v. Wiesner even though we fed monkeys with large amounts of the virus for 42 days. The results were negative, even when all of the mesenteric lymph nodes, which could be removed by operation, were taken out before feeding was begun. At the end of the experiment, the monkeys were shown to be just as susceptible to intracerebral inoculation of the virus as were normal monkeys. It becomes important to repeat these experiments as Aycock (17, 114) has recently reported three small outbreaks of poliomyelitis which he believes can be traced to infected milk.

Distribution of Virus in Monkeys with Experimental Poliomyelitis

Early in the course of the experimental inoculations of monkeys it was found that the virus is not present exclusively in the infected regions of the spinal cord but occurs also constantly in the brain (73). Later (60) the virus was found to be present in the intervertebral ganglia of infected monkeys. Flexner, Clark, and Amoss (86) found the virus also in the Gasserian and abdominal sympathetic ganglia. Landsteiner (125) showed that the virus is not stored in the peripheral nerve but seeks the parenchymatous nervous organs in which to multiply. But the selection among the latter appears to be small, since the virus occurs regularly not only in the spinal cord, but in the brain and the several ganglia mentioned of which some are remote from the cerebrospinal axis. It has likewise been found many times in the mesenteric lymph nodes in monkeys and human beings (78). The localization of the virus in the ganglia takes place early. Monkeys in the preparalytic stage of infection and early in the state of excitement, before any detectable weakness of muscles has set in, already show lesions of the intervertebral ganglia. The histological changes in the ganglia correspond accurately to those in the spinal cord: they are partly interstitial and perivascular, partly parenchymatous. The former extend in from the pial investment of the ganglia to the fibrillar supporting tissue and to small blood vessels which may be the seat of diffuse or of nodular accumulations of mononuclear cells. The degree to which the nerve cells are destroyed is variable. At times they escape entirely even when the interstitial changes are marked; again they are markedly degenerated, or even necrotic, and have become invaded with neuronophages. It appears as though an extension of the cellular invasion takes place from the subarachnoid spaces of the spinal cord. The changes in the Gasserian ganglia are less severe and those in the abdominal sympathetic ganglia are still less marked. The virus is not found in the blood after the usual routes of inoculation. In a single instance among ten tests reported by Clark, Fraser, and Amoss (38) infection was secured with a specimen of blood removed at the beginning of the paralysis on the seventh day following an intracerebral inoculation. When suspensions of the spinal cord from a paralyzed monkey have been injected into the brain, or simultaneously into the brain and spinal canal, the blood removed from 1 to 48 hours later fails to cause paralysis after intracerebral injection. When large volumes of active filtrate are injected into the circulation, the blood remains infective for at least 72 hours, but may be no longer

infective after 10 days when the paralytic symptoms first appear. When, however, the filtrate is injected in smaller amounts, or when a filtrate of a less active virus is employed in large quantity, the blood even fails to convey infection to the brain and cord, or conveys it irregularly. It is only when overwhelming quantities of an active virus are injected into the blood that paralysis results. The injection of moderate doses is not followed by paralysis, although the virus may still be detected in a sample of blood taken 24 hours after the injection. The existence of a mechanism capable of excluding the virus within the blood from the central nervous organs is therefore inferred (59, 66). Following intravenous injection, for about the same period (72 hours), the virus may be found also in the various lymph glands of the body or in the bone marrow (60). After the ordinary route of infection, even though the monkey dies of experimental poliomyelitis, the virus is not found in the bone marrow. After intravenous injection, it is likewise found in the spleen but not in the kidney. The spinal fluid has not been found to contain virus during the prodromal or acute stage of the disease. It is found, of course, in the spinal fluid for a few days following an intraspinal or intracerebral injection, or following the intravenous injection of large amounts of the virus. After death from experimental poliomyelitis, regardless of the route of injection employed, the virus is found in the nasal secretions, nasal mucosa, tonsils, and lymphoid tissues of the pharyngeal ring (80).

Distribution of the Virus in Human Cases

The occurrence of virus in human tissues corresponds to the distribution in the monkey inoculated by the usual routes with the exception of intravenous injections. It has never been found in human cerebrospinal fluid or in blood taken at various times during the acute stage of the disease. It is found in the nasal mucosa, nasal secretions, tonsils, adenoid tissue, and mesenteric lymph nodes, in addition to the various nervous tissues referred to in the discussion of the distribution of the virus in monkeys.

In 1912, Kling, Wernstedt, and Pettersson (111–113) reported positive results in monkeys inoculated with filtrates of the stools from cases of poliomyelitis. Unfortunately their criteria of experimental poliomyelitis are not generally accepted by other workers in this field and some doubt has been cast on their conclusions by Flexner and Amoss (60).

Pathogenesis

Flexner and Lewis (81) were able to infect monkeys by intranasal applications of the virus and to follow the course of the virus to the olfactory lobes. Since the first symptoms and signs of the disease in man and monkeys are due to pathological changes in the posterior root ganglia, one seeks the route of travel from the atrium to the relatively remote posterior root ganglia. The most logical route is by way of the cerebrospinal fluid, but the virus is present there in such small amounts that its progress cannot be followed by inoculation tests. Flexner and Amoss (59, 66) in working on this problem injected very large doses of the virus intravenously and tested the cerebrospinal fluid. No virus was detected in the fluid after expiration of 48 hours; only small amounts at the expiration of 72 hours; following an expiration of 96 hours the virus had passed more freely into the cerebrospinal fluid. The virus was still detectable in the fluid at the onset of paralysis, 19 days after the intravenous injection. When smaller doses are used intravenously or when a small amount of the filtrate is injected intraspinally, the virus disappears within a few days and is not present in the cerebrospinal fluid at the beginning of the acute stage of the disease. After the intravenous injection of even large amounts, the virus passes inconstantly from the blood to the tissues of the spinal cord and brain. The intervertebral ganglia are capable of removing the virus directly from the blood and indirectly from the spinal fluid. Aseptic inflammation produced by an intraspinal injection of horse serum facilitates and insures the passage of the virus from the blood to the central nervous organs, and the consequent production of paralysis. One presupposes therefore the existence of a mechanism of defense, so-called meningeal choroidal complex, which prevents the passage of the virus from the blood to the cerebrospinal fluid when intact, but, when this mechanism is broken down by an aseptic inflammatory reaction, the virus penetrates easily. The virus in transit from the blood through the cerebrospinal fluid to the sections of the spinal cord and brain is capable of being neutralized by intraspinal injection of immune serum. In their discussion of the pathogenesis of poliomyelitis based on such experimental evidence, Flexner and Amoss (60) say in part:

"It is now sufficiently obvious that the virus possesses affinity for nervous tissues in general, but for no element of these tissues in particular. The constancy with which meninges, blood vessels, interstitial parts, and nerve cells are affected indicates that they all react to the presence of the virus. On the basis

of actual observations it cannot be stated that virus is attracted by the nerve cells, either alone or necessarily in advance of the other structures mentioned; while the experiments here recorded show that it is only when the virus is brought to the nervous organs otherwise than by the general blood that the tissues composing them are able readily to remove and attach it to themselves.

"This latter fact is a cardinal point, and one from which we may derive valuable information on the pathogenesis and mode of infection of the disease.

"It may be regarded as established that all intraneural means of infection are successful, and that the virus travels with more or less ease and certainty along the nerves to the interstices of the central nervous organs, probably utilizing the lymphatic channels of communication. The experiments given in this paper show that the central nervous organs, excepting the intervertebral ganglia, are incapable of removing the virus from the general blood prior to changes induced in the blood vessels and in the choroid plexus. They indicate, also, that in the monkey these preliminary lesions are of a nature that permits of differentiation from the lesions caused by the intraneural modes of infection. The lesions in human cases of poliomyelitis would seem to correspond with those caused by intraneural and not by intravenous inoculation.

"In general it should be stated that the intraneural modes of inoculation are effective in proportion to the degree with which they bring the virus into intimate relation with the central nervous tissues. On that account intracerebral inoculation is the most effective, because it not only insures contact between the virus and the mechanically injured tissues, but also because it isolates the virus in the brain tissue, under conditions favorable to multiplication and gradual diffusion into the ventricles and cerebrospinal fluid. Intraspinous injection is somewhat less effective for the reason that a part, and sometimes perhaps all, of the virus may be carried into the general blood before it can reach and become attached to the nervous tissues. Intranasal infection is, in keeping with the general statement made above, more certain in its results than subcutaneous or intrasciatic inoculation, because of the proximity of the short olfactory nerve fiber to the brain tissue. It may fail, because the virus is washed away before it reaches the olfactory fibers and can be carried to the brain. Possibly intraocular inoculation may prove among the most successful, because the virus cannot escape and has only a short distance to travel to the brain; while the vitreous humor may even prove a favorable medium for its multiplication.

"In the main, under natural conditions, it is the upper respiratory mucous membrane that would most often become contaminated with the virus, and most readily favor its conveyance to the brain. This series of events is determined by the manner in which the virus is thrown off by the infected body, by the fact of its presence upon the nasal mucosa, even in healthy persons in contact with cases of poliomyelitis, and by the demonstrations that it passes, on the whole easily, along the olfactory nerve fibers to the brain, medulla, and spinal cord. Although the virus is conveyed to the nervous organs from without by the lymph, the distribution throughout the nervous system is, in large part at least, effected through the medium of the cerebrospinal fluid. . . . . This important fact has been established, not only by the finding of the virus by inoculation tests in the cerebrospinal fluid after a blood injection, but also through the prevention of infection

by the injection of immune serum into the subarachnoid spaces after lumbar puncture following the intravenous infusion of the virus under condition insuring infection.

"Thus the experimental evidence, which is upheld by observations in human cases of poliomyelitis, supports the view that epidemic poliomyelitis is caused by the entrance into the body of its specific microbic cause or virus, through the upper respiratory mucous membrane to the olfactory lobes of the brain, from which by means of the cerebrospinal fluid it is distributed throughout the substance of the nervous organs; but, since the virus may reach the brain by way of any nervous channel, and even, although with great difficulty, from the blood, it is, of course, possible that in exceptional instances other modes of infection may arise."

IMMUNITY

Active Immunity

An attack of poliomyelitis produces a definite and enduring immunity. There are no instances on record of a second attack in man (40). Lovett (personal communication, 1918) observed one patient whom he believed suffered a second attack. In the epidemic in Westchester County, 1916, we saw a lad, 5 years old, who experienced a mild febrile illness followed by partial foot drop and 9 weeks later died of the ascending type of poliomyelitis. Unfortunately the spinal fluid was not examined during the first illness. It is highly probable that second attacks do not occur.

Flexner and Lewis (77) were unable to reinfect monkeys which had recovered or were recovering from the disease. Out of more than 1,800 monkeys used at The Rockefeller Institute only two showed evidence of a sudden extension of the paralysis, following the subcutaneous injection of large doses of the virus several months after the first course of experimental poliomyelitis in efforts to produce hyperimmune serum.

Natural Immunity

The low attack rate in man will be commented upon under the section on epidemiology. Among the monkeys employed in the experimental studies, including *Macacus rhesus*, *Macacus cynomolgous*, *Macacus nemestrinus*, *Cercopithecus*, and the Philippine monkey, only in rare instances were the animals refractory to intracerebral injections of the virus (77). Human beings and monkeys are the only animals definitely known to be susceptible to poliomyelitis.

EPIDEMIOLOGY

Communicability

Wickman (244), as a result of a careful epidemiological study of the Swedish epidemic of poliomyelitis came to the conclusion that the disease is spread by transmission from person to person and that the so-called abortive (non-paralytic) form played a highly important part in its dissemination, and recognized that in some instances a healthy intermediary or carrier might be the medium of transmission.

The virus of poliomyelitis enters the central nervous system by way of the nasopharynx, and leaves the body by the same route. In recovered cases the virus probably disappears (64), except in rare instances (196) within 10 days to 2 weeks after the acute attack. The duration of carriage of the virus by healthy contacts has not been determined. Taylor and Amoss (238) found the virus in the nasal washings from a girl 6 days before the onset of a definite attack of poliomyelitis.

Field observations lead the author to believe that only slight contact between the carrier of the virus (case, healthy carrier, or person in the incubation period) and the susceptible person suffices for the transfer of the virus. In this respect, and also in regard to the fact that the virus is present in the nasal washings of the person at least 6 days before onset of symptoms, poliomyelitis resembles measles.

The stage of communicability, then, is from 6 days before to 10 days after the onset—in a general way, from 1 week before to 2 weeks after onset. The difficulties of preventing poliomyelitis by isolation are obvious.

Morbidity

Age.—Poliomyelitis is essentially a disease of young children, but children and adults of all ages may be affected. In the New York epidemic of 1916 (205), in which there were 9,005 reported cases, 97 per cent of them were among children under 16 years, and 73 per cent of 5,432 carefully studied cases were among children between 2 and 5 years of age. In Vermont (31), which is essentially a rural population, only 90 per cent of the cases were among persons under 21 years of age. At present there is no explanation for the high percentage of cases among adults of rural populations.

Attack Rate.—Even in large epidemics where it may be presumed that the virus is widely distributed, the percentage of susceptible individuals

is remarkably low. For example, in the epidemic of 1916, the attack rate for the total population was 3.8. The rate among persons over 10 years of age was very much lower, *i.e.*, 0.23. Because of the low attack rate it was formerly believed that only one case of poliomyelitis occurred in any family. In the New York epidemic (205), however, two cases occurred in each of 290 families. There were 17 instances of three, 2 of four, and 2 of five cases in one family. The author in the same epidemic in Westchester County, New York, observed four cases in a family of five children. The reason for the low attack rate is not clear, but three sets of experimental evidence have a bearing on this problem: Amoss and Taylor (9) found that the nasal washings from adults and from some children contained substances which neutralized or rendered the virus of poliomyelitis non-infectious for monkeys. The neutralizing principle was not found in the nasal washings of persons with chronic respiratory infection or during the acute stage of rhinitis. Obviously, if the virus should happen to be implanted upon the mucous membranes of individuals who possess this neutralizing principle in their nasal secretions, it would become innocuous before entering the body. If the virus was able to cross this first line of defense, there remains the next line of defense, *viz.*, the intact mucous membranes. Flexner and Amoss (68) found that the permeability of the nasal mucosa of monkeys was increased by the chemical irritants usually employed in nasal douches. The third line of defense is the intact meningeal choroidal complex already mentioned. It will be remembered that Flexner and Amoss (59) found that experimental poliomyelitis is induced with great difficulty by the intravenous injection of virus even when large amounts are given. If, however, an aseptic inflammatory reaction is set up in the meninges by the injection of serum or salt solution, much smaller amounts of the virus when injected intravenously suffice to produce the disease. The results are similar and analogous to those obtained when the virus is applied to the nasal mucosa. For example, if a pledget of cotton moistened with the virus is inserted into the nares of a monkey and allowed to remain only 2 hours, experimental poliomyelitis rarely results, but, if the monkey received the preceding evening an intraspinal injection of serum or salt solution, the virus comes into more intimate relation with the central nervous system and experimental poliomyelitis results.

Another mechanism decisively influencing the susceptibility of individuals to poliomyelitis is the presence of immune bodies in the serum, as shown by neutralization tests. Anderson and Frost (13, 14) noted the presence of immune bodies in the serum of 66 per cent of the persons

who had been in close contact with poliomyelitic cases but who had not contracted the disease. They suggest the possibility that persons in contact with acute cases may become actively immune. Flexner and Amoss were not able to confirm their observations in a study of twenty samples of serum taken from persons who were in close contact with cases of poliomyelitis.

Other factors may play a supporting rôle in deciding the susceptibility or non-susceptibility of individuals to the disease as is indicated by the fact that large doses of x-rays increase the susceptibility of the monkey to experimental poliomyelitis (10), and that Zingher (255) has observed positive Schick reactions in poliomyelitic children to be twice the expected rate for children of the same ages among the general population.

Insect Vectors

Howard and Clark (105) found that the domestic fly can carry the virus in an active state for several days above the surface of the body and for several hours within the gastrointestinal tract. Mosquitoes and lice did not in various experiments take up and maintain the virus in the living state. However, the bedbug was found to ingest the virus with the blood from the infected monkeys and maintain it in a living state within the body for a period of 7 days. In 1912, Rosenau and Brues (218), reported several instances of successful transmission of experimental poliomyelitis by means of the biting stable fly, *Stomoxys calcitrans*, a fact that was at first quickly confirmed by Anderson and Frost (14). The second series of experiments conducted by Anderson and Frost (15) were totally negative as were the comprehensive and critical experiments carried out by Sawyer and Herms (225). Clark, Fraser, and Amoss (38) were unable to confirm the results of Rosenau and Brues.

Milk-Borne Infection

Aycock (17, 114) has recently studied one American and two English outbreaks of poliomyelitis in which he believes that the etiological agent was transmitted by infected milk.

Persistence of the Virus in the Nasopharynx

Flexner and Amoss (68) showed that the virus occurs constantly in the nasopharynx of human cases, and of monkeys (80) having the experimental disease. In the human beings it has been detected in nasopharyngeal washings from acute cases, rarely in similar washings from healthy contacts (89), in the nasopharyngeal tissues obtained from fatal

cases during the first week of infection, but rarely, if ever, from nasopharyngeal tissues removed surgically at later periods in the course of the disease (64). In monkeys the virus has also been detected in the secretions from acute cases, in the nasopharyngeal tissues derived from early cases (68, 72), and rarely from animals several weeks or months after recovering from the acute symptoms (161). They deduced from these experiments that the virus is regularly present in the nasopharynx in cases, especially fatal ones, of poliomyelitis in the first days of illness, that it quickly diminishes in amount as the disease progresses, except in rare instances, and that it is unusual for a carrier state to be developed. It is possible that the early development of immune bodies in the blood of monkeys (as early as $3\frac{1}{2}$ days after onset of symptoms) and in human cases (fifth day of the disease) have a bearing on the disappearance of the virus from the nasopharynx.

Seasonal Incidence

There is a very definite seasonal variation in the incidence of poliomyelitis. In most of the epidemics recorded the disease makes its appearance in the latter part of June, reaches its peak during the last week of August or first 2 weeks of September, and declines rapidly in October with a few scattering cases in November. Aycock has shown that there is a small peak in May or June presaging a summer outbreak.

There have been a few winter outbreaks. Wickman (243) cites such an epidemic occurring in the north of Sweden, enduring through the winter, and reaching its maximum in April and May. He also reports three closely related epidemics occurring in neighboring sections of the country; the first (eighteen cases) lasted from June to October, the second (twenty-seven cases) from July to December, and the third (sixty-two cases) from the end of September to February with its maximum in November and December. In West Virginia an epidemic has been described which began in December and reached its peak during January and February. The occurrence of epidemics during the winter months may be considered as evidence against the hypothesis that poliomyelitis is spread by an insect vector such as the stable fly (*Stomoxys calcitrans*).

Carriers

The definite seasonal variation in the incidence of poliomyelitis and the experimental data supporting the view that the virus does not survive long outside the host suggest the recovered case, or the carrier, as

the interepidemic reservoir of the virus. The rapid disappearance of the virus from the nasopharynx as the acute stage passes, and the coincident development of immune bodies in the blood controvert the idea that the recovered case harbors the virus during the interepidemic period. However, the observation of Lucas and Osgood (161), *viz.*, finding the virus in the nasopharynx of monkeys 5 weeks and 6½ months after onset of the infection, is not to be disregarded. In experiments with monkeys, we have shown that the virus applied to the nasal mucosa of monkeys which have been passively immunized quickly disappears without causing experimental poliomyelitis, whereas in the normal monkey the virus persists (68). Therefore, all things considered, it seems that the virus has a greater chance of surviving in the nasal secretions of the healthy contact than in the recovered case. In the former there are only local agencies for combating the virus, whereas in the latter all the agencies of the body may react for defense. In spite of the very obvious experimental difficulties, the virus has been detected in the nasal mucus of healthy contacts (89, 111).

Isolation

The patient and intimate contacts are quarantined for 3 weeks. A search is made for all persons, especially children, who were associated with the patient during the week previous to the onset of the disease. The families of these contacts are then visited by a representative of the Board of Health who discusses the danger of repeated exposure. Thus children who may be in the incubation period are voluntarily quarantined for 2 weeks. The efficiency of quarantine varies inversely with its duration. It is suggested, therefore, as a practical measure that the period of isolation for cases and contacts be reduced to 17 days from onset.

Prophylaxis

Quarantine.—Quarantine measures, such as used in Vermont with apparent success, should be observed. During an epidemic, travel and visiting should be limited to absolute necessity. The breadwinner should keep away as much as possible from other members of the family, especially children, and should observe the rules of good respiratory manners.

Antiseptics.—Various antiseptics, used in nasal douches, have been recommended as preventive measures. Experiments with monkeys have shown that these procedures not only possess no value in preventing the

disease but actually increase the liability to infection (68). It has been found that rinsing with isotonic salt solution mechanically removes the bulk of the virus in carriers and promotes the neutralizing power (9) of the nasal secretions. It should be remembered, however, that some risk of sinus infection attends such procedures.

Active Immunization.—Flexner and Amoss were unable to actively immunize monkeys by repeated injections (215) of dried virus according to the Pasteur method. But Aycock and Kagan (20) have recently published suggestive results. Flexner and Amoss (69) described a strain of poliomyelitic virus derived from a rapidly fatal human case, which exhibits mild degrees of infective power and marked degrees of protective effect for the monkey. While the *Macacus rhesus* displays perceptible differences in susceptibility to this unusual virus, nearly all individuals respond to the inoculation and none succumbs to the infection induced. Compared with previously described examples of experimental poliomyelitis, this type of the disease is distinguished by its relatively benign nature and its tendency to end in recovery rather than in death. No experience has been had in the active immunization of man against poliomyelitis.

Passive Immunization.—The injection of human convalescent serum is not recommended for two reasons: (1) it is so difficult to obtain and so precious that it should be reserved for treatment, and (2) the attack rate is so low that it would be necessary to immunize large numbers of children on the chance of protecting the few susceptible individuals. There is no test for susceptibility. As has been stated, it is unusual to find more than one case in a family.

TREATMENT

Convalescent Serum

Since the lesions of poliomyelitis are in the most remote and inaccessible parts of the body, the treatment of the disease is a difficult and almost hopeless task. At present, serum treatment[2] seems the most logical one to use. In the absence of any immune animal serum, recourse must be had to convalescent human serum which is obviously weak in antibody content when compared with a hyperimmune serum such as is employed in the treatment of meningitis. Serum from recently recovered cases is recommended, as it is presumed to have more

[2] Treatment with hexamethylenamine (urotropin) or with adrenalin has not given encouraging results.

antibodies at this time than later. The basis of serum treatment rests upon the observation of Römer and Joseph (184, 215) that immune bodies are present in the blood of recovered cases (185). The experiments of Flexner and Lewis (82) show that the injection of such serum delays and may altogether prevent the development of paralysis in monkeys previously inoculated with the virus. Netter (187) was the first to treat human cases with convalescent serum.

Collection of Serum from the Donor.—Obviously the donor should be free from infectious diseases and the blood Wassermann negative. In epidemics, the author has collected serum from suitable cases 6 or 8 weeks after the onset of the disease.

As much blood as can be safely withdrawn (from 200 cc. to 500 cc. according to the weight of the patient) is collected in sterile centrifuge tubes or convenient sterile flasks. The operator should direct the stream of blood so that there is the minimum amount of damage to the red cells. The blood is allowed to clot and stand at room temperature overnight. The serum is drawn off, centrifuged, and inactivated at 56°C. (132.8°F.) for ½ hour. After tests for sterility, it is preserved with 0.35 per cent tricresol, centrifuged, and placed in 20 cc. bottles which are kept in an icebox. Samples of serum from several sources when available are given consecutively. In emergencies, blood may be withdrawn from the donor into a sterile flask containing sufficient sodium citrate (chemically pure) to prevent coagulation, and immediately centrifuged. The plasma is used only for intravenous injection.

Amount of Serum Used.—Since only weakly immune serum is available, large amounts of it must be given. Obviously the amount of serum that can be injected intraspinally is limited, and Draper (44) has called attention to the severe reactions which follow the use of large amounts. On account of this limitation and for other reasons about to be described, the author has employed combined intraspinal and intravenous injections (5). Flexner and Amoss (65) found that poliomyelitic antibodies in the blood would pass into the spinal fluid if the meninges were inflamed, and Amoss and Eberson (8) showed that the disappearance of antibodies from the spinal fluid in meningitis proceeds at a slower rate if immune serum is also injected intravenously.

The procedure of administering the serum is as follows:

After withdrawal of the spinal fluid the needle is left inserted and the lumen stopped with the stylet so that no more fluid escapes. Samples of the fluid are immediately examined microscopically and chemically. If the results of the examination of the fluid and clinical picture warrant the diagnosis of acute poliomyelitis, 15 to 20 cc. of serum are immediately given very slowly intraspinally. This method saves time and a second lumbar puncture in positive cases. The

patient is then placed in a comfortable position and from 50 to 100 cc. of serum are injected intravenously. The injections are repeated after 12 hours in severe cases. If the symptoms are aggravated and the respirations become irregular, or rapid and shallow following treatment, another lumbar puncture should be made immediately and the excess fluid withdrawn.

Results of Therapy with Convalescent Serum.—The results of several observers are suggestive, but Peabody (199) believed that the value of convalescent-serum therapy has not been established. Since it is common knowledge that the severity of outbreaks of poliomyelitis varies considerably, treated cases should be compared only with untreated controls in the same epidemic. Draper (44), who has had a wide experience with poliomyelitis, believes that a cell count in the spinal fluid above 100 indicates a serious type of the disease. In the series treated by Amoss and Chesney (5), there were several cases of this kind, in which the cell count was high. They responded favorably to serum treatment. The results of these workers, when analyzed, show definitely that the progress of the disease was arrested in cases treated within 48 hours after onset with more than 50 cc. of serum. They believe that the remainder of this series may serve as partial controls. Ulrich (240) reviewed the reports to 1917 concerning treatment, called attention to the lack of controls in the series reported by Amoss and Chesney, and stated that their series is of little value. Some of Ulrich's patients were treated by the subcutaneous injection of the patients' own spinal fluid! Zingher (255) employed normal and immune serum and prefers the latter.

Hypertonic Solutions

It seems logical to suppose from the histological picture and from the fact that in some cases a complete paralysis will disappear or improve markedly in a short time that perineuronal edema plays an important rôle in the causation of paralysis. If by some means the edema could be lessened until the natural defenses of the host are mobilized, one might expect some beneficent result from such a procedure. Since Weed and Hughson have shown that the central nervous system can be shrunk by dehydration following the intravenous injection of hypertonic salt solution, Aycock and Amoss (18) sought to apply this method to the treatment of poliomyelitis. In a small series of monkeys the results were suggestive of a beneficent effect.

Since the conditions for the re-formation of edema would remain in the perineuronal spaces, they attempted to saturate the host with im-

mune bodies by intravenous injection of convalescent serum after the dehydration had time to take place in order that when the edema returns immune bodies might be carried into the locus of inflammation. Their hypothesis has not been definitely proved experimentally due to the expense of monkeys, but they have treated a child in the acute stage of ascending paralysis (Landry's type) which is so often fatal and the child recovered (19). Dr. T. Cook Smith in Park's Clinic in New Haven treated a patient of the same type with hypertonic solution and thought there was distinct improvement. Obviously no conclusions can be drawn until many cases have been treated, especially since they often clear up without any treatment. It is definite that the volume of the central nervous system in human poliomyelitis is rapidly diminished following the intravenous injection of 20 to 25 cc. of hypertonic Ringer's solution (usual formula without carbonate multiplied by 20). With this assistance twice as much immune serum may be injected intraspinally.

The procedure is as follows:

As much fluid as possible is withdrawn by lumbar puncture and replaced by convalescent serum until no more runs in. While the needle remains in place, an intravenous injection of 25 cc. of the hypertonic solution is injected very slowly under light anesthesia. The immune serum in the lumbar puncture funnel will soon begin again to flow into the canal. Five hours later, 50 cc. of convalescent serum is injected intravenously.

Hyperimmune Serum

Flexner and Lewis (77) were unable to produce experimental poliomyelitis in any animal except the monkey. Amoss and H. D. Taylor made repeated subdural injections of the virus in sheep, goats, horses, calves, and donkeys and did not succeed in making an antipoliomyelitic serum.

Pettit (201), Etienne (48, 49), and Etienne, Stroup, and Benech (50) have made reports concerning a hyperimmune serum (made in horses and sheep) for poliomyelitis. From the data at hand it is impossible to draw any conclusions as to the value of the serum.

Rosenow (220), and Nuzum and Willy (195) in the belief that certain streptococci bear a causal relation to poliomyelitis immunized horses with these organisms. They state that such immune horse serum possesses neutralizing, protective, and therapeutic properties in poliomyelitis. Their neutralization tests were made *in vitro*. This method has been found to give inconstant results. Amoss and Eberson (6, 7) tested samples of these sera by the *in vivo* method based on the power of conva-

lescent serum to neutralize the virus in the cerebrospinal fluid as it comes through as a result of aseptic inflammation.

Their procedure was as follows:

Multiples of three monkeys were given an intraspinal injection of 2 cc. of normal horse serum in the afternoon. The following morning each received one intravenous injection[3] of 50 cc. of a paper filtrate of a 5 per cent suspension of fresh poliomyelitic tissue. Immediately intraspinal injections were made. Monkey 1 received 3 cc. of normal horse serum; Monkey 2, 3 cc. of convalescent poliomyelitic serum (either from monkeys or human cases); the third monkey, 3 cc. of the serum to be tested, activated with 1/10 volume of fresh guinea-pig serum. The intraspinal injections were repeated daily for 3 days. Monkey 1 succumbed to experimental poliomyelitis, Monkey 2 survived. In the test with Rosenow's and with Nuzum and Willy's serum the monkeys succumbed. These sera did not show any neutralizing power *in vivo*. The latter serum was tested for neutralizing substances *in vitro* and for therapeutic properties with negative results.

It may be concluded from the experimental evidence at hand that there is no hyperimmune serum for the treatment of poliomyelitis and that the so-called antipoliomyelitic sera, which are really antistreptococci sera, have no specific action in poliomyelitis.

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[3] Suspensions from fresh poliomyelitic tissue should be employed for intravenous injection because autolysis takes place rapidly. Suspensions of autolyzed nervous tissues markedly lower the blood pressure and the monkey may die during the injection. In some experiments with Cash in 1923 (unpublished), it was found that these depressant substances may be removed to within the zone of safety by allowing the suspension to percolate slowly through permutite. By this process, the virus is not removed from the suspension.

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VIRUS DISEASES OF MAMMALS AS EXEMPLIFIED BY FOOT-AND-MOUTH DISEASE AND VESICULAR STOMATITIS

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CHAPTER VI

VIRUS DISEASES OF MAMMALS AS EXEMPLIFIED BY FOOT-AND-MOUTH DISEASE AND VESICULAR STOMATITIS

A discussion of foot-and-mouth disease is included in this book because it is the first disease of animals that was shown to be induced by a filterable or ultramicroscopic agent. Moreover, the virus has received such widespread attention that it may be regarded as the most thoroughly studied one of this group of infectious agents. A brief discussion of the virus of vesicular stomatitis of horses is also included, inasmuch as it is similar in many respects to the virus of foot-and-mouth disease. Furthermore, it received but scant attention until the recent work of the American Commission[1] and of the writer. These studies serve as bases for its taxonomy.

In the following pages it is impossible to give a complete résumé of the literature on foot-and-mouth disease. Nor is this indicated. The reader who is especially interested can find complete reviews in the authoritative and comprehensive articles of Hutyra and Marek, of Gins and Krause, and of the British Committee.[2] It is the writer's intention, however, to discuss the important phases of the problem of foot-and-mouth disease and to aid in this manner the student who de-

[1] This Commission to Study Foot-and-Mouth Disease was formed by the United States Department of Agriculture, Bureau of Animal Industry, and comprised Dr. Harry W. Schoening, of the Bureau, Dr. Jacob Traum, of the University of California, and the writer, of The Rockefeller Institute for Medical Research. Dr. Louis Boëz, of the Institute of Hygiene, Strasbourg, France, where most of the work was carried on, acted as collaborator. The carrier problem was studied at Alfort, France, with the assistance of Professor Vallée. The entire Commission was under the direction of Dr. John R. Mohler, Chief of the Bureau of Animal Industry.

[2] The present British Foot-and-Mouth Disease Research Committee (Chairman, Professor C. J. Martin) was formed by the Ministry of Agriculture and Fisheries in 1924. Investigations have been carried on mainly at the Ministry's Veterinary Laboratory, New Haw, Weybridge, England, and at its Experimental Station, Pirbright, England, under the direction of the late Sir Stewart Stockman. Studies on the virus were also made at the Lister Institute of Preventive Medicine, London, under the supervision of Dr. J. A. Arkwright. To date two reports have been issued (see bibliography).

sires to compare them with similar phases of problems of other ultramicroscopic virus diseases.

Discovery of the Ultramicroscopic Character of the Virus

It was a chance finding by Loeffler and Frosch (1897), and not a deliberate experiment which led to the discovery that the virus of foot-and-mouth disease is filterable and ultramicroscopic. In studying the problem of immunity, they attempted to free vesicular contents (so-called "lymph") from corpuscular elements by passing the "lymph" through Berkefeld filters. The filtrates were found to be as typically active in cattle as was unfiltered material. From this point it is interesting to note their mode of reasoning in arriving at the conclusion that the virus is a multiplying, invisible agent.

They first considered the possibility of the filtrate being a toxin. Accordingly, a unique system of titration was employed. A specimen of lymph was diluted 1:15,000 gms. A unit of this diluted lymph, injected into a 200 kg. calf, produced a typical affection. Since the "poison" was distributed evenly throughout the body, a final dilution of 1:15,000 $\times$ 200,000, or 1 part of filtrate to 3,000,000,000 parts of calf, was active. Similarly, 1 gm. of filtrate to 7,500,000,000 gms. hog's blood was infective. Since tetanus toxin is active in rabbits and guinea pigs in dilutions no greater than 1:1,000,000 and 1:1,000,000,000 respectively, they realized that the activity of this filtrate exceeded that of known toxins and that the filtrate was probably not of the nature of a soluble toxin. "On the contrary," they say, "it is in effect the result of a multiplying agent. The smallest bacteria known to the present are Pfeiffer's—which are 0.5 to 1 μ long. Let us suppose that the incitant of foot-and-mouth disease is $\frac{1}{10}$, or perhaps even $\frac{1}{5}$ as large, which is not an impossibility." Then it is invisible by means of the ordinary microscope. However this is, the fact that the virus is ultramicroscopic has since been confirmed by many workers throughout the world.

Cultivation of the Virus

The consensus of opinion at the present time is that morphologically distinct bodies, identified as the incitants, are not cultivable from foot-and-mouth disease materials, when a lifeless, artificial medium is employed. The inciting agent, therefore, conforms in this respect with the general character of ultramicroscopic viruses outlined by Dr. Rivers in the first chapter.

A large variety of formed elements, however, has been advanced by several investigators as the incitants of the disease. Gins and Krause, in reviewing the literature to 1923, recount the following as having been regarded at one time or another as the causal agents of foot-and-mouth disease: *Oïdium albicans*, a fungus (Hadinger, 1864); *Tilletia aphthogenes*, a fungus (Bender, 1869); a micrococcus (Rivolta, 1876); an ascomycete (Babes and Proca, 1897), a coliform bacillus (Siegel, 1891); a streptococcus (Kurth, 1893); a coccus (Lermusan, 1893); a pleomorphic bacterium (Stutzer and Hartleb, 1897); a typhoid-like bacillus (Starcovici, 1897) and a pleomorphic bacillus (Niessen, 1897). A number of protozoa also have been implicated as the incitants. Among them are: *Sporozoön aphthæ epizoöticae* (Behla, 1893); *Protamoeba aphthogena* (Piana and Fiorentini, 1894); *Cytorrhyctes aphtharum*, a coccus-like sporozoön (Siegel, 1905); a protozoön resembling eosinophilic granules (Terni, 1909); sporozoa (Liebes, 1913); and *Aphthomonas infestans*, a trypanosome (Stauffacher, 1915). None of these findings has been definitely confirmed by others. Indeed, histopathological examination of tissues has either failed to reveal these forms, or has shown that they are non-specific elements produced by inflammation (Loeffler and Frosch, Stauffacher, v. Betegh, Huntemüller, American Commission, etc.).

More recently, artificial cultivation, by means of which no formed bodies were discernible, merely a multiplication of the virus, has been described by Titze (1924), by Pfeiler (1924), and by Guth (1924). In some of these instances successful transplants were characterized by clouding of the medium. Titze found that he could maintain the virus in his cultures for 4 successive subplants in a beef serum broth medium. While the subplants were not pathogenic, they were antigenic: hogs could be solidly immunized with them. Pfeiler, however, reported that his virus maintained its virulence for 34 subplants, but an absence of details of his methods has prevented a general acceptance of his results. In respect to Titze's work, the American Commission has shown that it is unwise to conclude that activity of the virus in the first few transplants indicates multiplication, inasmuch as the virus is infective in a dilution of 1:10 million. In regard to clouding of the medium, it is well known that this character is no constant indicator of sparse growth or growth of minute microörganisms. Olitsky and McCartney (1923) have discussed at length the pitfalls of precipitate production in serum medium. It appears, therefore, that the virus has not as yet been successfully cultivated in a lifeless medium when the usual methods of culture were employed.

Still more recently, in 1924, Frosch and Dahmen created widespread interest when they reported successful cultivation of the virus by employing a new mode of procedure. Their cultures consisted of minute, bacillary bodies, *Loeffleria nevermanni*. The colonies, as well as the bodies themselves, were visible only by means of monochromatic, short wave length, ultraviolet photomicrography. Their method of cultivation consisted in centrifuging the lymph for a long time in order to concentrate the virus, and then washing the sediment in changes of salt solution to free it from so-called inhibiting substances. The sediment was seeded on a solid medium, containing Martin broth, 3 per cent agar, to which was added, just before use, 20 to 50 per cent of horse or ox serum. The medium was adjusted to pH = 7.8. Growth occurred aërobically after 7 to 10 days at 33°–34°C. Suspensions of these subvisible colonies, even to the 25th subplant, induced in guinea pigs experimental foot-and-mouth disease, although sometimes the reactions were quite weak.

The German Commission directed by Gins, the British Committee, and the American Commission have failed to confirm the work of Frosch and Dahmen. In accordance with the statements of the latter, prolonged centrifugation of the virus suspensions was a prerequisite for successful cultivation. All commissions have found that this step in the technique did not concentrate the virus, nor could the so-called inhibiting bodies be removed in this way. Furthermore, it was found that the virus failed to survive in the Frosch and Dahmen medium after 3 days.

The writer and others have shown the optimum conditions for the preservation of the virus in artificial media to be as follows: The hydrogen ion concentration of the medium should not vary from the narrow range of 7.5 to 7.6 (confirming the results of the British Committee) throughout the period of observation. A strict anaërobic atmosphere appears to be advantageous, as well as a temperature below 37°C. A semisolid structure of the medium is favorable and this can be effected by the use of 0.25 per cent agar, or 10 per cent gelatin. Of the two, gelatin is more desirable, and of the latter the most effective preparation is gelatin prepared after the manner of Loeb in studying its isoelectric condition. In this material the impurities have been removed thoroughly. Adjustment to the proper hydrogen ion concentration is made with potassium hydroxide. Gelatin is the simplest of protein media available, and its use is in keeping with the principle that the virus requires only a simple material for life: the addition of organic or complex protein substances, such as dextrose, broth, serum, lipoids, etc., to a

simple basic medium causes destruction of the virus. It is thus not surprising that the American Commission was unable to confirm the results of Frosch and Dahmen concerning the cultivation of the virus, for neither their medium nor their method satisfies the essential conditions necessary to maintain artificially the life of the virus. The deductive study of its requirements for artificial life made by the American Commission indicates that the metabolism of the incitant is of a very simple kind and is limited to definite and inflexible conditions. Therefore, in respect to cultivation the virus appears to be of an order wholly different from that of known cultivable microörganisms.

In the foregoing discussion the failure of artificial cultivation of the virus applies only insofar as lifeless media have been employed. By means of living tissue cultures, the American Commission obtained only inconclusive results with the virus of foot-and-mouth disease. The writer, with Dr. Carrel, has studied the problem anew in America, using the virus of vesicular stomatitis which bears a close generic relation to that of foot-and-mouth disease, and it was found that the former apparently multiplies considerably in tissue cultures. The hope is entertained that similar results may be obtained by others with foot-and-mouth disease. If, in the future, the virus withstands artificial cultivation in lifeless media and yields only to living tissue cultures, it should then be regarded as an obligate parasite, requiring the living cell for its multiplication, either within or without the body.

Properties of the Virus

The properties of the foot-and-mouth disease virus, which serve as a basis for its taxonomy, will be described. The reader will observe that these characteristics are wholly distinct from those which are usually regarded as necessary for a taxonomic study of cultivable bacteria.

Activity of the Virus.—The British and the American Commissions have found the limit of infectiousness of the virus to be at a dilution of 1:10 million. The writer has also observed that Berkefeld filtrates of materials containing vesicular stomatitis virus are practically as infectious as are specimens of the original unfiltered suspensions of the active agent. There are several considerations of importance in connection with the activity of the virus in extraordinarily high dilutions. In the first place, it is correlated with extremely minute size of the virus, since the titrated materials, or filtrates of them, do not consist of virus alone. Secondly, virulence maintained only in the first few subplants in experiments on cultivation should be interpreted as probably indicat-

ing a mere survival of some of the original virus rather than its multiplication. Thirdly, not all samples of virus are equally infectious. Therefore, comparative studies should be made with the same sample. Furthermore, since very minute quantities of the virus are able to induce infection, one should be extremely careful to avoid errors arising from contaminations with the active agent.

Centrifugation.—It has been determined by Abe, Gins, the British and American Commissions that the virus is not sedimented by centrifugation: after centrifugation at from 3,000 to 5,500 R.P.M. for 1 or 2 hours, the topmost layers are usually as infectious as the lowermost. Here again non-centrifugability may be an indication of the minuteness of the size of the incitant. Duclaux and other physicists have shown that centrifugation is not an adequate means for the deposition of minute particles (see Olitsky and Boëz, 1927, I).

Resistance to Chemicals.—The virus of foot-and-mouth disease is remarkably resistant to a number of chemical reagents, notably those of the fat-solvent group, alcohol, ether, and chloroform. Loeffler and Frosch found that diluted vesicle fluid containing 1 per cent phenol was active for at least 5 months. Abe stated that 70 to 75 per cent alcohol precipitated the virus along with the albumins of infective blood which then retained its activity for 2 or 3 days in a dried state. Stockman and Minett reported that 25 to 50 per cent alcohol did not destroy the virus within a period of 3 days, and 10 per cent within a period of 20 days. In the Second Progress Report of the British Committee, it is further reported that the virus, at 5°C., resisted 50 per cent glycerol for at least 400 days, and 96 per cent for 10 months. Moreover, it withstood saturation with sodium chloride, sodium or magnesium sulphate for 3 months in a cold room. The virus also resisted the action of sodium fluoride, toluol, phenol, lysol, hydrogen peroxide, chlorine, iodine, chloropicrin, chloroform, ether, and acetone in concentrations which rapidly destroyed ordinary bacteria. They found, however, that the incitant was killed within 1 hour by antiformin, 1 per cent.

We have confirmed this extraordinary resistance of the virus to some chemicals. On the other hand, we have shown that this resistance operates only under limited conditions. This is due to the fact that certain reagents coagulate the proteins of the medium in which the virus is usually suspended. As a result of the coagulation, the positive charge of the virus, and its minute size, the virus is held within the large coagula and is kept from direct contact with the chemicals. On the other hand, if advantage is taken of the periodic phenomenon attend-

ing coagulation, or if coagulation is prevented (as, in the case of alcohol, by adding a trace of sodium hydroxide), the virus is then acted upon directly by the antiseptics. Under these conditions, the incitant is as sensitive to destruction by the reagents as is the living staphylococcus. Among the reagents which do not form coagula are sodium hydroxide and antiformin. A 1 per cent solution of either reagent kills within 1 minute the virus in vesicular contents, in bits of tissue, in material admixed with cattle manure, urine, or garden soil. It cannot be stated definitely, therefore, that the resistance of the virus to chemicals is an indication of its inanimate character.

Electric Charge.—We have found the electric charge carried by the virus to be positive and by cataphoresis tests have shown that its isoelectric range is at the high point of pH = about 8. With regard to these properties, it may be said that the virus differs from ordinary bacteria. The common genera of bacteria carry an electronegative charge and have a much lower isoelectric point. The fact, however, that the virus has an unusually high isoelectric range and is, under ordinary conditions, electropositive does not speak against its possible protein or living nature. For, although bacteria are generally electronegative, spirochetes, as a genus, carry electropositive charges (with the exception of *Treponema pallidum*); so do trypanosomes, such as the species of *brucei*, *equiperdum*, *gambiense*, and *rhodesiense*. Among protein substances, fibrin and gliadin are known to possess a high isoelectric point.

Filterability.—The virus of foot-and-mouth disease passes through earthenware filters more readily than does vaccine virus, thus resembling more closely the viruses of mosaic disease and chicken plague, and the bacteriophage.

In this case, as in that of all ultramicroscopic viruses, a proper interpretation of filtration phenomena is dependent on a knowledge of the principles involved. These have been discussed in the first and second chapters. The points of importance in considering the results of filtration of the virus of foot-and-mouth disease are: the amount and nature of the diluent, the time necessary for the completion of filtration, the degree of pressure used, the electric charge of the virus and the filter, the composition of the filter, the use of material containing free or unadsorbed active agent, and the control testing of the filters with particles of known charge and size. In regard to the latter, control tests with particles of known charge and size are of utmost importance in employing the ordinary collodion filters; for microscopic holes in such filters frequently result in passage of the virus, a phenomenon unrelated to the actual capacity of the incitant to pass intact collodion.

It appears that the virus is easily filterable through one asbestos disc of Seitz' filter and through Berkefeld "V" candles. It does not traverse all the Berkefeld "N" candles used. The virus also passes freely through Chamberland L 1 to L 5 bougies, but is retained in some L 7 and L 9 bougies. In the L 11 type, under ordinary conditions, complete adsorption of the electropositive virus in this electronegative filter prevents the appearance of the former in the filtrate, but, if the charge of the virus is changed so that it is electronegative the filtrates are infectious. The American Commission was unable to pass the virus through collodion membranes (confirming the work of the British Committee) unless Bechhold's ultrafilter membranes were employed, and of these, the most permeable types.

Size.—Access to a number of filters of different pore sizes and to tested control materials of known charge and size led us to a consideration of the possibility of delimiting the dimensions of the incitant. Such tests are impracticable if all of the virus in a medium is adsorbed to other particles. But we could not convince ourselves that the virus was thus adsorbed. That the virus can be free and that there is a lack of complete adsorption to a number of different substances has been observed also by other investigators (Dahmen, Gins, Bedson, Burbury and Maitland, and Stockman and Minett). Furthermore, filtration proceeds in a definite, regular manner, and there is practically no loss in activity of the virus after filtration. For the above reasons, and from the results of cataphoresis experiments showing a separation of the virus from protein particles of the medium in which the virus is suspended, one cannot regard the whole of the incitant as adsorbed only to minute particles of the same size. A purely relative measurement, by a system of so-called "molecular" filtrations through Bechhold's ultrafilters, showed the active particles of the foot-and-mouth disease virus, in relation to other particles of like charge, to be between 20 and 100 millimicrons in diameter.

Adsorption of the Virus.—We found that the virus could be adsorbed to the visibly large, soft, flocculent coagula of protein. Vallée and Carré believed that the virus could be completely attached to red blood cells or killed bacteria, and consequently removed from its medium. The British Committee has been unable to confirm their results. Gins also failed to adsorb the virus by means of kaolin. Dahmen, using charcoal, was unsuccessful. Abe, on the other hand, states that he has succeeded with both substances. It is obvious that this virus adheres with much more difficulty to standard adsorbents than do other ultramicroscopic incitants.

Effect of Temperature.—In blood or in vesicular fluid, the virus loses its activity at 37°C. after 24 hours. Loeffler and Frosch reported that the virus in the fluid was inactivated at 30°C. in 9 days, and at icebox temperature after 8 months. These results have been confirmed by several investigators, and it may be concluded that some substance is elaborated or activated in virus suspensions outside the body at a moderately high temperature which inhibits or kills the virus. What this is has not as yet been determined. In this regard, it is interesting to note that in living tissue cultures, the closely related virus of vesicular stomatitis can survive, or even multiply, at a temperature of 39°C. for at least 8 days. This corresponds to what occurs in the living animal during infection. At 50°C., the virus is killed in 15 minutes (Loeffler and Frosch); at 55°C., the virus in defibrinated blood is rendered inactive in 20 minutes, and in filtered, diluted vesicular fluid in from 15 to 40 minutes (British Committee). At 60°C., vesicular contents are inactivated in 5 minutes (Trautwein).

Effect of Light.—The British Committee has found that the virus of foot-and-mouth disease is sensitive to sunlight. The writer, with Dr. Gates, has studied a phase of this problem with the virus of vesicular stomatitis, and has shown that the reactions of staphylococci to measured amounts of monochromatic energy at various wave lengths correspond to those of the virus. These parallel reactions are indirect but suggestive evidence that the substance of the virus is similar in chemical constitution to bacterial protoplasm, if the absorption of specific energies is an index of chemical character.

Effect of Drying.—Roux and his coworkers reported the survival for 105 days of rapidly dried virus, a fact later confirmed by Vallée. Stockman and Minett have recently extended the period to 112 days, when the virus was dried on glass slides over sulphuric acid at room temperature. Burbury has shown a much greater resistance of the active agent under similar and other conditions. Thus it appears that the virus can be quite resistant outside of the body, contrary to certain opinions that it is then very fragile.

Survival of the Virus within and without the Body.—The virus is usually present in the blood during the initial febrile stage of the disease. In the experimental disease in guinea pigs, it may be found in the circulation from 6 to 14 hours after cutaneous injection (Ernst, Goebel, and v. Seigneux). This experience is correlated with that of Krause who was able to prevent generalization of the virus by excising the infected area of the skin 4 hours after inoculation. While ordinarily the incitant

disappears rapidly from the blood of cattle, workers in Gins' laboratory have shown that in exceptional cases the virus was still present in the blood of experimentally inoculated guinea pigs 19, 22, 34, and 198 days after cutaneous injection (Gins and Weber, Krause, Fortner). This finding is significant in the recognition of possible carrier infections.

The virus is also present in the fluid and in the coverings of the vesicles. Under these conditions, it is much more concentrated than in the blood. The results of the American Commission's investigations showed that in the vesicles of cows and hogs the incitant was active for guinea pigs for not longer than 7 days after the first appearance of symptoms. The repeated findings of others indicate that the virus may be present at one time or another in practically all secretions and excretions, although the question has not as yet been answered as to whether these materials are admixed with vesicular contents. The American Commission, however, has shown that cattle harbor the virus in their oral secretions before the first symptoms of the disease are manifest.

Important and extensive studies have been made by the British Committee on the survival of the active agent in carcasses of infected guinea pigs, cattle, and hogs. They found that, in carcasses of guinea pigs, blood was infective after 36 days, bone marrow after 96 days, and epithelium from the lesions after 102 days. In carcasses of cattle and pigs which had been slaughtered in the early stages of foot-and-mouth disease, dressed for market, and either chilled or frozen, blood was infective for 30 to 40 days and bone marrow for 76 days. Aside from the epizoölogical importance of these findings, they reveal the extraordinary resistance of the virus and its remarkable viability in bone marrow.

The viability of the incitant outside the body is dependent upon the conditions under which it is kept. As we have already indicated, cold and darkness, as well as the narrow range of pH 7.5 to 7.6 of the medium, favor its survival. Hecker reported that the virus remained alive on hay for 2 months. Bedson, Maitland, and Burbury, of the British Committee, extended this period to at least 15 weeks; and to at least 20 weeks in the case of bran. They found the incitant on cow's hair after 4 weeks. The American Commission found that it remained viable for at least 25 days in moist or palpably dried garden soil. Recently Trautwein reported that it survived in manure for 41 to 67 days.

In conclusion, it may be stated that the virus of foot-and-mouth disease, similar to some other ultramicroscopic viruses and also to certain bacteria, shows, under definite conditions, a considerable resistance to destruction both *in vivo* and *in vitro*. Much attention has been given to this subject here because of its epizoölogical importance.

Plurality of Types.—A great advance has been made in research on foot-and-mouth disease by the discovery by Vallée and Carré (1922) of the presence of two types of the virus, the "O" and the "A." This discovery has since received ample confirmation. Waldmann has recently described three distinct types, "A," "B," and "C." The presence of at least two types of virus is of the utmost significance to the efficacy of control measures, to epizoölogical conditions, and to investigations on immunity. These types do not induce cross-immunization, as determined by tests on cattle, hogs, and guinea pigs, nor do they produce a clinical picture by which one can be distinguished from the other. While the presence of differing types is usual among visible bacteria, this has not as yet been proved to be common in the group of ultramicroscopic viruses.

In respect to the virus of vesicular stomatitis of horses, it is important to note that the recent studies of Cotton show that there are two types of this virus also. Both types produce identical clinical pictures, but animals recovered from the effects of one are not immune to the action of the other.

Respiration.—The active agent could not be separated in a pure state from respiring, living host tissues, nor could it maintain its activity under the conditions imposed by a respiration experiment. Hence no definite results could be obtained (American Commission) in regard to respiration of the virus.

It will be noted from the description of the properties of the foot-and-mouth disease virus that nothing has been brought forward to the present time to indicate that the virus is of an inanimate character or that it is of the nature of a *contagium vivum fluidum.* Nor has any fact based on direct evidence been adduced to show that the incitant is a living, multiplying agent. On the other hand, a number of findings, indirect and suggestive to be sure, have been noted which support the notion that the incitant may be living. This question of the animate or inanimate character of the virus remains to be answered definitely by future observers.

Susceptibility of Man and Animals

Practically all cloven-foot animals are susceptible to the virus of foot-and-mouth disease. Horses, mules, and asses are apparently resistant to its effects. In nature, the disease is one of the most contagious known. Its rapid spread is exemplified by the recent epizoötic in California. "The outbreak extended to sixteen counties and, within six

months, more than 100,000 animals were destroyed in bringing the pestilence under control" (Mohler[3]).

Clinically, especially in cattle and hogs, the disease begins with high fever 24 to 48 hours after exposure. As a rule, distinct vesicles occur on the feet in 48 hours. The animals become lame and move about with difficulty, often walking on their knees. Vesicles may also be present on the tongue or in the mouth, and in cows, on the udders. The animals are very sick and as a rule do not eat for several days. There is great loss of weight and cows retain their milk or produce a scanty, discolored, unmarketable fluid. Within a few days, however, the temperature returns to normal, the animals begin to eat, the feet and mouth are less sensitive, and healing takes place gradually. The convalescence is protracted for several weeks, during which time a distinct line of separation forms between the new and old horn, and new epithelium gradually covers the eroded areas in the mouth and on the udders. In some animals a localized necrosis of the myocardium occurs—whether this is a secondary reaction is still questioned. In general, this virus shows the same tendency of other viruses to invite secondary pyogenic infections.

Transfer of the Virus to Guinea Pigs.—Waldmann and Pape (1920) and, independently, Hobmaier succeeded in regularly producing experimental foot-and-mouth disease in guinea pigs. Waldmann and Pape's important results have since been amply confirmed by many workers. The importance of this work depends upon the fact that the disease in these rodents is a counterpart of that in bovines. Primary vesicles appear at the point of inoculation within 24 to 48 hours after cutaneous injection; these are followed in a day or two by secondary vesicles in the uninoculated mucosa of the mouth, on the tongue, or in the pads. Thus the guinea pig offers an aid to diagnosis and to laboratory studies on the virus, and is suitable for its propagation. Attention should be given to the fact, however, that some types of virus, particularly Vallée and Carré's "A," are not readily adaptable to guinea pigs or are wholly non-pathogenic for these rodents (British Committee). Moreover, there is no difficulty in inducing foot-and-mouth disease in cattle and hogs with guinea-pig virus. The affection so induced is similar to the natural disease. While in nature the disease is highly contagious,

[3] The reader who is interested in the epizoölogy and control of this disease is referred to Mohler, J. R., Foot-and-mouth disease, *U. S. Dept. Agric.*, Circular 400, Washington, Dec., 1926. Here will be found a clear, comprehensive, and impressive account of the spread and suppression of the California (1924) and Texas (1924–25) epizoötics.

there is practically no spontaneous communicability among guinea pigs (British and American Commissions). It is interesting to note that inoculation of the virus in sites other than the hairless pads may yield some information concerning epizoötics and immunity. After an appropriate inoculation of virus in the pads, by scarifying and "tunneling" the epidermis, practically all guinea pigs react with the experimental disease. In those, however, which are injected intramuscularly, intraperitoneally, subcutaneously, or intracutaneously in the abdominal skin, from 20 to 65 per cent fail to show demonstrable lesions. When such lesions do occur, they are only found in the pads or on the tongue, never at the site of injection (American and British Commissions). Thus, the indication is that the virus is peculiarly epitheliotropic and probably has a limited portal of entry into the animal's body.

Transfer of the Virus to Other Small Animals

Rabbits.—Gins and Fortner found that rabbits could be infected by smearing guinea-pig virus on the scarified mucous membrane of the inner lip surface. Vesicles appeared, and the contained fluid was in turn active for normal rabbits and guinea pigs. Sixteen consecutive rabbit passages were thus effected. Nicolau and Galloway were able to induce local vesicles after intralingual injections of the virus. In neither case was there evidence of secondary lesions or of generalization of the process. The experimenters of the British Committee induced lesions with the virus after injecting it into the tongue of rabbits. Nine passages were successful and in the later passages vesicles were noted in the mouth. Hence the rabbit appears to be susceptible, but, in view of the irregularity of the occurrence of secondary lesions, it is not as suitable as the guinea pig for laboratory studies. Burbury has shown, in addition, that natural infection is rare in rabbits (and "wild rats") in captivity.

Dogs and Cats.—These animals do not show the usual clinical evidences of the experimental disease, although a high mortality rate and ulceration on the tongue can be induced, and scrapings from the ulcers are infectious for guinea pigs. The persistence of virus in the mouths of these animals is of short duration—2 days (British Committee).

Rats and Mice.—In the wild rat, no evidence of adaptation of the virus is detected. In white rats, inoculation of the tongue with guinea-pig virus results in several successful consecutive passages. Only primary vesicles are observed. White mice appear to be naturally immune, but

field mice can be infected with great regularity (Buschle, British Committee).

Hedgehogs.—This species is apparently susceptible when the virus is introduced intramuscularly and by scarification of the pads (British Committee).

Birds.—The British investigators have found birds to be insusceptible to the virus.

They conclude (First Progress Report) that none of the small animals mentioned can be implicated in the spread of foot-and-mouth disease either under laboratory or field conditions.

Susceptibility of Man.—The virus of foot-and-mouth disease is active for man, as is another ultramicroscopic incitant of animal origin, rabies virus. The affection in man is similar to that in animals, except that it is, as a rule, much milder and practically non-lethal. Moreover, occurrences are rare and spread from man to man is not the rule. The accidental infection of Dr. Pape, who was wounded in the hand by the breakage of a glass container filled with vesicular contents, confirms the fact that man is susceptible. In Pape's case, there was a short incubation period, onset with fever, exanthem on the hands and feet, and rapid healing (Gins, 1924). Gins explains the absence of lesions in the mouth by the fact that the patient was inoculated in the hand. But cases with vesicles in the mouth have been reported (Gerlach) when the source of infection was known to be milk or milk products, the usual carriers of the virus.

Carriers

The carrier problem has received considerable attention, but the subject is still in an experimental stage. Flies could not be implicated as vectors (Lebailly). Experiments with bugs were also unsuccessful (Bedson, Maitland, and Burbury). At one time birds were considered possible carriers, but the British Committee found no proof that this is the case. Earthworms (American Commission), field mice, and other rodents (Buschle, British Committee) cannot be implicated. Mohler (1926) was able to infect guinea pigs with ticks fed on affected cattle, but he cautiously indicates that the virus may have been passively transferred in the blood which distended the insects.

With respect to recovered cattle acting as carriers, the American Commission was first to put this problem to experimental test. Twenty selected, recovered Swiss cattle, presumed to be possible carriers of foot-and-mouth disease virus by the Swiss federal veterinary officials, were placed in contact with 28 normal cattle and 4 hogs of French origin, and

7 normal cattle from Switzerland. About 1 month before this experiment was terminated, the hoofs of the suspected carriers were pared and scraped, so as to make the test more rigid. During the 3-month contact with the possible carriers, no disease occurred in the normal animals, and at the end of this period all the normal cattle were successfully inoculated with "O" and "A" types of the virus. In another series, the hoofs of 22 cattle and one hog were examined at post mortem 20 days to 6 months after the onset of the disease. The hoofs were sectioned, and pockets, double soles, and the other tissues about the feet were scraped. The scrapings were then inoculated into guinea pigs. The material from only one of the possible carriers, slaughtered 34 days after inoculation, yielded positive results. After consideration of both tests, it was concluded that in a very few instances a recovered animal may harbor the virus. This number is apparently much smaller than that believed by others. On the other hand, it may explain in part the isolated outbreaks due to carriers reported by Italian, British, and Swiss officials.

The survival of the virus in hoofs for at least 34 days in a recovered animal lends support to the idea discussed in the first chapter in regard to the prolonged retention of viruses in the body, even in fully recovered cases. Foot-and-mouth disease may be looked upon as a good example of a disease in which this occurs. Illustrative of this, Loeffler believed cattle to be infectious 6 months after recovery. The Swiss federal officials institute an 8-month quarantine, although they believe that the virus may be retained by recovered animals for a longer time. Bang (quoted by Hutyra and Marek) reports the case of a bull that was infectious $2\frac{1}{2}$ years after recovery. To these instances may be added (1) the experience of Fortner who found the virus in the blood of dead guinea pigs 198 days after inoculation, and (2) the persistence of virus in tissues of carcasses, as shown by the experimenters of the British Committee. While the examples of retention of the active agent in living tissues are isolated and infrequent, yet the conclusion is warranted that the virus may be maintained in a supposedly healthy organism for a long time.

In respect to man as an actual carrier in the sense of his harboring the virus in his tissues or secretions, Kling and Höjer have recently advanced the notion that the virus resides in the mucous membrane of the respiratory tract. This idea lacks confirmation (Magnusson). Man is known, however, to act as a passive conveyor of the virus by its adherence to his clothing, hands, etc.

At this point it may be stated that the virus of foot-and-mouth disease may exist in symbiosis with vaccine virus (Mohler and Rosenau, Belin) and at least one epizoötic was traced to this source. It is of interest to note that there is some suppression of the effects of the foot-and-mouth disease virus during this symbiosis.

Pathology

The gross and microscopic pathology in large animals is similar to that observed in the experimental disease in guinea pigs. It is interesting to note that, apart from the local vesicular lesions and enlargement of regional lymph nodes, the gastro-intestinal tract may be involved. The stomach is congested and empty and contains mucus. Blood may be found in the duodenum and jejunum and ulcerations may occur in the large intestines. This is the case, however, only in the more virulent type of infection. The spleen and liver are apparently normal, but the adrenals may be injected and show small hemorrhages (Gins). The lungs are edematous and the nasal mucosa reveals catarrhal inflammation. The pericardial fluid is increased. For 4 to 5 days after injection there is a leucocytosis of polymorphonuclear neutrophiles. The virus is not neurotropic (Levaditi). To what degree the changes in tissues other than the skin are due to invasion of secondary microorganisms is still unknown.

The lesions in the skin and mucous membranes are similar in certain respects to the vesicular lesions induced by other ultramicroscopic viruses. The changes in experimental foot-and-mouth disease, comprehensively described by Nicolau, Alberta-Lorente, and Galloway, differ, however, from those of herpes febrilis when the virus of the latter disease is injected into guinea-pig pads. In herpetic infections, lesions develop more slowly, requiring about 4 days for the appearance of vesicles. There is considerable edema of the tissues. The lesions extend more deeply and tend more to coalesce. Furthermore, the vesicular lesions rapidly become pustular. Finally, no secondary vesicles are seen (Gildemeister and Herzberg). We have noted, however, that, following pad injections with Flexner and Amoss' H.F. strain of herpetic virus, gangrene often developed, and that paralysis or paresis of the posterior extremities associated with complete retention of urine and feces frequently occurred.

The nuclear changes observed in cells affected by the virus are of particular interest. Gins has described intranuclear inclusion bodies which occur especially in 24-hour old vesicles of the tongue. They are

small, about 1.5 microns in diameter, perfectly round, acidophilic bodies which are sometimes surrounded by a basophilic rim. A nucleus may contain one or more of these corpuscles. Gins stated that these are characteristic of foot-and-mouth disease. Trautwein and Ruhle, however, do not agree with Gins, inasmuch as they reported that they were able to find similar inclusions in tissues manifestly free from the virus of foot-and-mouth disease.

Rivers and the writer have noted bodies resembling Gins' so-called inclusions in early vesicular lesions of foot-and-mouth disease and of vesicular stomatitis. In addition, especially in lesions 48 hours old, intranuclear inclusion bodies were found occasionally, differing from Gins' structures, which are similar in some respects, but not necessarily identical with those noted in certain other virus diseases, *e. g.*, herpes. Briefly, they are round or oval and about 2 microns in diameter. With Giemsa's or eosin and methylene blue stain, they appear homogeneously pink to red in contradistinction to the blue nuclear membrane, and usually lie on a clear background. Some nuclei may contain 2 or 3 of the smaller-sized bodies. Hence, foot-and-mouth disease and vesicular stomatitis can be placed in the group of ultramicroscopic viruses, the effects of which are characterized by the presence of peculiar intranuclear changes in affected cells. The ultimate significance and importance, however, of these nuclear changes observed in the two diseases under discussion rely upon further investigations.

Immunity

The recent discovery of at least two types of the foot-and-mouth disease virus which show identical clinical pictures but no cross-immunity has served to obscure the results of prior work on immunity. The reported findings concerning the duration of the immunity have indicated, in the main, that it is relatively short. Loeffler believed that the insusceptibility persists about a year. Waldmann and Trautwein indicated that the time is much shorter. Moreover, in the field, a number of cases have been observed to show that an animal could be reinfected several times during the course of a year. How many of these repeated infections are due to the operation of a different type of virus is now unknown. On the other hand, Gins reported an instance in a guinea pig in which the immunity persisted for at least 387 days; Eichhorn, from 5 to 7 years; Siedamgrotzky, from 1 to 10 years. Arduenghi described a case in which resistance lasted for 8 years, and Schunck, one in which it lasted for 9 years.

With respect to recent work on this problem, Stockman and Minett (Second Progress Report), aware of the plurality of types, found that in recovered cattle a solid resistance to reinfection is maintained for at least 13½ months. This experiment is still in progress. Yet with reference to guinea pigs, in this report (p.19) it is stated: "When these animals have passed through an attack they remain resistant for a variable period, usually three to four months, occasionally longer. . . . Later, as the immunity passes off, not only local vesicles, but also secondary vesicles in the mouth and on the uninoculated feet follow intradermal inoculation of a large dose." It must be remembered, however, that what applies to guinea pigs may not hold true in cattle. It is obvious that the question of the duration of immunity requires further study, but for the moment, and until further work definitely establishes a fact to the contrary, one may conclude that the duration of immunity in foot-and-mouth disease differs from the solid, permanent resistance which characterizes many other ultramicroscopic virus diseases.

Types of Immunity in the Experimental Disease.—Serum from recovered cattle and guinea pigs, or hyperimmune serum does not prevent the occurrence of primary lesions. Such serum only prevents the development of a generalization of the disease, as indicated by secondary vesicles. The results are the same if the serum is injected simultaneously with virus in one area, or if it is inoculated shortly before or after the virus at different sites. Soon after recovery, a guinea pig may be completely resistant to reinfection. Later, however, resistance is evident only against secondary vesicles and not against local lesions (British Committee).

The findings described above have led Terni, Waldmann, and others to speak of two types of immunity, local (tissue) and general (humoral). In view of the fact that it has not been decided whether the two types are different in kind or in degree, the British workers call them "partial" and "complete" immunity. To support the opinion that these processes are only different in degree, the experiments of Waldmann and Trautwein, and of Krause may be cited. The former found that a second cutaneous inoculation of virus in swine failed to produce vesicles at the site of injection when it was administered 48 hours after the first inoculation, the time of appearance of the local lesions resulting from the primary infection. The latter reported that intratesticular injections of virus in guinea pigs, although followed by no demonstrable lesions, rendered the animals "completely" resistant. It is generally recognized, however, that the local or tissue immunity is first to disappear after re-

covery of an animal (Waldmann and others). Recently Minett published that immunity is due essentially to the presence of antibodies in the serum; and there is no evidence for the existence of a cellular immunity confined to cutaneous epithelium. He showed that an amount of virus smaller than is usually employed when injected into the skin can convert a "partial" into a "complete" resistance.

Active Immunization.—It has been the goal of workers on the problem of foot-and-mouth disease to find a means of actively immunizing animals. After considerable experimentation, the American Commission confirmed the opinion of others that "avirulent material does not induce immunity, and there is no method devised to the present (1926) by which attenuated or active virus can be employed for immunization without first causing manifest lesions."

Since then, however, the hopes of workers have been raised by Vallée, Carré, and Rinjard's experiments concerning the protective action of "formalinized vaccine." Their vaccine consisted of a virus emulsion treated with 0.5 per cent of formalin at 20°C. for 5 to 7 days. It was innocuous for guinea pigs after intradermal inoculation. Furthermore, it was found that type "O" vaccine protected 4 of 27 guinea pigs against type "O" virus, and that type "A" vaccine protected 10 of 19 guinea pigs against the homologous virus. Cattle inoculated intramuscularly with 0.3 gm. of epithelium were rendered resistant to a second subcutaneous injection of 10 cc. of the homologous type of active virus given 26 to 31 days later.

The British Committee independently studied this subject at about the same time. In the preparation of their vaccine, a different technique, requiring the use of only 0.1 per cent formalin, was employed. It was found that one injection of the vaccine in guinea pigs was followed by a "partial" immunity, that is, it was capable of protecting the animals against secondary vesicle formation, but not, as a rule, against the primary lesions. If 3 doses of the vaccine were given, "complete" protection was afforded against 10 to 100 minimal infecting doses. Furthermore, one injection of formalinized vaccine, followed by subinfective doses of living virus, induced complete immunity to several thousand minimal infective units of virus (Bedson, Maitland, and Bury). Cattle were not protected by small doses of the vaccine. At present the subject of dosage is being investigated with the hope that the vaccine may yet prove of value in field work.

Hyperimmunization. Serum Therapy.—The attempts of the workers of the American Commission to hyperimmunize cattle and a horse by

repeated injections of virus failed to produce more potent serum than that of convalescent animals. Indeed, serum from the horse was absolutely of no protective value, in spite of the fact that horses are insusceptible to foot-and-mouth disease. (Compare with remarks concerning immunity in the first chapter.) On the other hand, hyperimmunized guinea pigs and rabbits yielded the most actively neutralizing serum.

Since its discovery by Loeffler and its subsequent modification by Waldmann, hyperimmune serum has been employed now and again, mainly in Germany, with some measure of success. Its present use as a preventive appears to be limited to cattle in transport to fairs and markets, and to the possible preservation of blooded animals. As a therapeutic agent it is, at times, remarkably effective. In general, however, the large amounts required,—1 cc. to each 5 kg.—the shortness of the period of passive immunity (average 10 days), the expense of the treatment, and the occasional failure to protect limit its usefulness.

Antibodies in Immune Serum.—The American Commission was unsuccessful in demonstrating complement-fixing antibodies in immune serum, although a large variety of antigens and sera were employed. Stockman and Minett also failed. Moreover, they were unable to obtain flocculation, employing the methods of Sachs-Georgi, Dreyer and Ward, Krück, and the colloidal benzoin and the formol gel tests. So also Hedson, Maitland, and Burbury could not disclose any antigen-antibody reaction by precipitation or clumping. The absence of such antibodies is of interest in view of the partial neutralization of active virus admixed with immune serum. Since certain ultramicroscopic viruses, *e.g.*, smallpox virus and vaccine virus (Gordon) lead to antibodies demonstrable *in vitro*, such as complement-fixing bodies and precipitins, the virus of foot-and-mouth disease differs, in this respect, from them and from many cultivable bacteria.

Virus of Vesicular Stomatitis

The American Commission (1926) referred to the similarity exhibited by the clinical picture of vesicular stomatitis to that of foot-and-mouth disease. During a recent, extensive epizoötic of vesicular stomatitis in Port Jervis, New York, and in the northern part of New Jersey, the clinical picture was again very similar to that of foot-and-mouth disease.

Comparison with the Virus of Foot-and-Mouth Disease.—Characteristics common to the two viruses, deserving special notice, are: ability to be propagated in guinea pigs in continuous series, clinical and pathological pictures of the experimental disease, the lack of cross-immunity

with herpes virus, the limit of infectiousness (1:10,000,000) of filtered or unfiltered virus suspension, relative size, inability to be sedimented by centrifugation, behavior towards chemical and physical agents, similarity in response to selective filtration, sensitiveness to changes of hydrogen ion concentrations of the diluent, and resistance to destruction outside the animal body. Moreover, in recent studies by Long and the writer, it was found that the virus of vesicular stomatitis acts similarly to that of foot-and-mouth disease after injections in the leg muscles or in abdominal skin of guinea pigs. Under these conditions the virus fails to yield manifest local lesions but does induce a solid immunity in the pads of these animals. Such discrepancies as the infrequency of secondary lesions in vesicular stomatitis have been ascribed to variability in the strains of virus, inasmuch as some strains of foot-and-mouth disease virus also behave irregularly in respect to production of secondary vesicles. In spite of the striking resemblance between the two viruses, there are certain clear-cut differences exhibited by them, such as lack of cross-immunity in guinea pigs, cattle, and swine, and the failure of the horse to respond to inoculation with the virus of foot-and-mouth disease while being highly susceptible to the virus of vesicular stomatitis.

It will be noted, therefore, that these two viruses differ practically only in the absence of cross-immunity in recovered animals and in the resistance of the horse to one of them. In view of the non-cultivability of both, it is difficult to prove the precise relationship of one to the other. Yet, if a comparison be made with the conditions existing in regard to plurality of types among the different strains of foot-and-mouth disease virus itself, or among known cultivable bacteria, such as the pneumococcus, a close generic relationship between the incitants of vesicular stomatitis and foot-and-mouth disease may be inferred. For example, there are at least two types of foot-and-mouth disease virus, either of which shows no cross-immunity to the other in recovered animals. While all strains are generally active in cloven-foot animals, there are some to which the guinea pig is resistant (British Committee, Vallée, and others). Here, then, is a genus of a virus containing types which do not exhibit cross-immunity and which show different pathogenic effects in certain species of animal. Analogous conditions are observed in dealing with pneumococci. Rabbits are susceptible to Type I and Type II pneumococci, but are usually resistant to Type III (Tillett). It is well known, moreover, that these types are different immunologically.

By way of conclusion, the writer has endeavored to show the relation-

ship of the viruses of foot-and-mouth disease and vesicular stomatitis to one another, to other ultramicroscopic incitants, and to known cultivable or visible bacteria. It will be noted that many analogues are found in common with subvisible active agents and with cultivable or visible microörganisms. The outstanding fact is that the viruses are not all alike from a taxonomic standpoint; they differ among themselves, just as one genus of bacteria differs from another.[4]

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[4] One should not close an article on foot-and-mouth disease without expressing admiration of the work of the personnel of the United States Bureau of Animal Industry, directed by Dr. John R. Mohler. Through its vigilance the country has been kept relatively free from infection for long periods of time and the spread of the infrequent epizoötics has been promptly checked.

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VIRUS DISEASES OF FOWLS AS EXEMPLIFIED BY CONTAGIOUS EPITHELIOMA (FOWL-POX) OF CHICKENS AND PIGEONS

ERNEST W. GOODPASTURE

CHAPTER VII

VIRUS DISEASES OF FOWLS AS EXEMPLIFIED BY CONTAGIOUS EPITHELIOMA (FOWL-POX) OF CHICKENS AND PIGEONS

INTRODUCTION

An eruptive disease of fowls, bearing a superficial resemblance to the pox of certain mammals, seems to have been observed from time immemorial. The exceeding infectiousness of the disease in the common fowl greatly impressed early Indian authors (Heusinger). It was described during the seventeenth and eighteenth centuries as affecting doves, hawks and turkeys. In earlier times when smallpox was so prevalent and devastating, the appearance of this disease of fowls no doubt assumed in the minds of the people an alarming significance because of the rather natural conjecture that it was variola, which, it was believed, could be transmitted from fowls to man (Burnet).

The supposition of an identity of fowl-pox, smallpox and vaccinia was later expressed in the writings of authors early in the last century (Schmid). This view, it seems, was first seriously questioned by Spinola (1858) for the reason that he did not succeed in transmitting cow-pox to birds, while certain other domestic animals were susceptible. Bollinger, being unable to infect a goat and a sheep with fowl-pox, also doubted its identity with variola and vaccinia.

The discovery by Rivolta (1869) of the curious "inclusion bodies" associated with the cutaneous eruption of fowl-pox was followed shortly by the histological studies of Bollinger (1873) who distinguished the lesion from that of variola and classified it with the tumors. Several years later (1892) came the demonstration by Guarnieri of characteristic intracellular bodies in the lesions of variola, differing from those of fowl-pox. The pathological distinctions between the two diseases then seemed so convincing that the unitarian theory of fowl-pox and smallpox appears to have been temporarily abandoned.

During the last quarter of the preceding century investigations of infectious diseases were centered upon a pursuit of etiological agents, and fowl-pox was subjected to the feverish search for specific bacterial and protozoan parasites. Along with certain other diseases of as yet

unknown etiology, it proved a rich field for the protozoa hunters of this period, particularly because of the demonstration of the rather ill-defined, though specific, cellular changes which have always afforded a basis for considerable morphologic speculation. Somewhat later, after the studies of Loeffler and Frosch (1898) on foot-and-mouth disease, its virus shared with those of several other infections the distinction of filterability, a property which was found to be an attribute of many viruses. About the same time that the filterability of fowl-pox virus was demonstrated by Marx and Sticker (1902), Centanni filtered the virus of fowl pest, Borrel that of sheep-pox, and Nicolle and Adil-Bey the virus of cattle plague.

From the point of view of cellular inclusions contagious epithelioma had already become more or less associated with molluscum contagiosum, variola and vaccinia. Soon it was to be drawn closer to these and other virus diseases, both by the property of filterability of its causal agent and the presence of specific cytologic changes. Negri discovered the inclusions of rabies in 1903, and the same year the virus was filtered by Remlinger. In 1905 Negri filtered vaccine virus and Juliusberg the virus of molluscum contagiosum. Other diseases fell rapidly into the same group.

With the demonstration of the filterability of certain viruses the idea of their invisibility suggested by Pasteur in 1881 for the active agent of rabies (Volpino) again found expression; but the studies of Borrel upon contagious epithelioma of pigeons in 1904 brought morphologic evidence of the possibility that this and certain other viruses might be microscopically demonstrable by proper staining methods. The cultivation by Nocard and Roux of the germ of peripneumonia and its morphologic study by Bordet, demonstrated the untenability of the hypothesis that a virus, because it happens to be filterable, must necessarily be ultramicroscopic.

Notwithstanding its temporary abandonment, the early view which identified fowl-pox, or epithelioma contagiosum as it was named by Bollinger, with variola and vaccinia has lately been resurrected, and has its advocates among modern students of the disease. Indeed investigations during the past 50 years have brought to light many similarities and analogies between the two infections, not only in respect of the eruptive nature of the exanthems, but in a resistance of the respective viruses to drying and to glycerol, their filterability, their curious affinity for epidermal structures, the occurrence of analogous changes within the cells of the lesions, and the establishment of a more or less permanent

immunity in the recovered animal. However, immunological facts relating to the specificity of the two viruses are tending to dispel the time-worn notion of their identity, and to allay the suspicion that the one may be transformed into the other, although the active agent of neither disease has as yet been cultivated on a lifeless medium. The relationship of the viruses of fowl-pox and of the human disease, molluscum contagiosum, seems at present a more inviting field for study.

The ready availability of the virus of epithelioma contagiosum, its high degree of infectiousness and the easy adaptability of the disease to laboratory investigation have made it a contributing factor of great importance in the accumulation of much of our knowledge of virus diseases in general, and it is believed that many facts of fundamental importance may yet be derived from its study. It is particularly adaptable to investigations of the significance of specific cellular inclusions and their relation to the nature and behavior of viruses, to studies of intracellular parasitism and immunity, and to efforts toward that great desideratum, successful cultivation of the active agent on artificial media. There are those indeed who would believe that the virus of epithelioma contagiosum has already been successfully grown, though curiously enough the investigators who are supposed to have accomplished this (Bordet and Fally) specifically deny it. The question centers around the problem of the identity of fowl-pox and avian diphtheria.

Although there seems no doubt that many of the infections now classified in the great group of diseases due to filterable viruses will be shown to be caused by bacterial or protozoan parasites, or possibly to non-living agents, it is the opinion of the writer that in final analysis the viruses of contagious epithelioma, molluscum, vaccinia, variola, rabies and certain others, which exhibit both the property of filterability and the capacity to induce specific cellular changes, will remain together in a much smaller, though a more closely related and compact group, whatever their essential nature may eventually prove to be.

Epidemiology

Epithelioma contagiosum of fowls, or fowl-pox, is a highly contagious, specific infectious disease endemic in many lands, and recurring not infrequently in epidemic form. Epidemics appear frequently in the summer, and also at other seasons (Csokor). The disease is more commonly seen in domestic fowls, frequently attacking an entire flock when

once introduced, and often resulting in a high mortality. The fowls that recover are immune to a subsequent infection.

Not only is the domestic chicken susceptible, but the disease affects also pigeons, turkeys, geese, pheasants, hawks and probably parrots. Little or nothing is known of the infection in wild birds, though it seems possible that the virus may occasionally be spread to domestic fowls by sparrows or other birds. Such an assumption does not rest, however, on experimental evidence. In a single experiment virus from an infected hen was inoculated by the writer upon the scarified skin of an English sparrow's breast with a negative result. In fact little is known of the relations of the various strains of the virus in fowls other than chickens and pigeons, since practically all of the experimental work has been done with these birds. Notable differences between the strain of virus adapted to chickens and that found in pigeons have come to light, namely that, as first recognized by Bollinger, it is easy to infect hens with the pigeon virus, but the reverse experiment rarely if ever succeeds. The pigeon virus is also susceptible to a biological modification by a single passage through the hen, inasmuch as its virulence for the pigeon is thereby greatly reduced, and may be completely destroyed (Marx and Sticker). This striking change in the virus is analogous, in the opinion of Marx and Sticker, to the modification of variola virus when inoculated upon calves.

The possibility of one species of fowl spreading the disease to another species would apparently depend upon the characteristics of virulence of the strain of virus concerned, which cannot be predicted *a priori*. Variation in virulence analogous to that of the hen and the pigeon strains may exist in each strain of the virus adapted to a different species of fowl.

Such differences of virulence as exist between the virus of pigeons and that of the common hen are not the only difficulties encountered in an attempt to explain the epidemiology of the disease, for there is at present nothing very definitely known about the manner of spread of the infection from one individual of a species to another. Contact infection, from intimate cohabitation of a normal susceptible hen or pigeon in the same cage with infected individuals of the same species under laboratory conditions, is rarely if ever seen (Burnet, Lipschütz), though the nodules of the abundant eruption and the dried scabs which separate from them are enormously rich in virus. Superficial injuries of exposed surfaces offer possible portals of entry for the virus under natural conditions, and Burnet has succeeded in infecting pigeons from which feathers had been

plucked over extensive areas, thus offering numerous skin wounds for reception of the virus, by placing them in contact with heavily infected cagemates. More suggestive is his occasional success in infecting pigeons by feeding them grain saturated with virus. No method of artificial infection, however, has as yet seemed adequate to explain the rapid and thorough infestations which occur under natural conditions.

Once the virus infests a barnyard it is difficult to eradicate it, as is indicated by the frequently quoted observation of Bollinger, who states that a poultry yard infested one autumn resulted in the extermination of the major portion of the flock. The fowls that survived the disease were killed. Following this, strenuous efforts were made to disinfect the environs. The fowls obtained the next spring were placed in another compartment about 100 yards from the previous one where the infected fowls had been kept. After a short time the disease reappeared, rapidly infecting the new flock. We now know that fowl-pox virus is one of the most resistant of infectious agents, withstanding drying, light and even moisture for long periods. This indicates a high degree of adaptability to natural outdoor conditions.

The fowls which have survived an attack of the disease may carry the virus for long periods, and possibly serve as sources for other outbreaks. No observation has come to the writer's attention as to the elimination of virus through the kidneys and the intestinal canal, though it is expelled from the fowl in enormous masses in the form of desquamated incrustations from the eruptions.

The Spontaneous Disease

After an incubation period of about 4 to 6 days the most characteristic clinical feature of the disease, the cutaneous eruption, appears usually upon the unfeathered parts of the body. It is observed particularly about the head, affecting the comb, wattles, ear lobes, eyelids, nares and angles of the beak. It may also appear on the legs and feet, under the wings and about the anus. The cutaneous exanthem is characteristically nodular. When first noticeable the nodules measure less than 1 mm. in diameter, appearing grayish or pink, circumscribed, firm and seemingly deep-seated. They may be very numerous or few in number; and they do not all erupt necessarily at the same time. Rapidly enlarging and becoming more elevated during a period of a week or more, they assume the appearance of warts attaining a size of about 0.5 cm. in diameter. Sometimes they remain more or less flat. Closely adjoining lesions may coalesce. The larger developing lesions appear

rough, gray, yellow or dirty brown in color. The surface may be moist for a short time but soon dries superficially. After about 2 weeks of development, sometimes sooner, the nodules may show evidences of inflammation at their base. They then frequently become hemorrhagic and begin to dry, assuming an irregular dark brown or black, scabby appearance. The process of desiccation and scab formation lasts for another week or two. If the crust be removed in the meantime a moist sero-purulent exudate is found beneath, covering a bleeding, granulating surface. Eventually the large, brown or black, hard, shrunken crust drops off leaving a smooth scar. In milder lesions there may be no noticeable scarring. The lesions about the eyelids are often very extensive, involving the margins of the lids, partially or completely closing the aperture. There may be an associated purulent conjunctivitis or keratitis with destruction of the cornea. At the angles of the beak the lesions usually become infected with microörganisms from the mouth and present a whitish or dirty gray, opaque, necrotic, flat surface which is moist and membranous. Neighboring lightly feathered parts of the head, and sometimes the upper part of the neck, may be affected. Reischauer mentions the occurrence of disseminated and generalized forms of the eruption affecting wider areas of the skin. In geese, lesions appear upon the webs of the feet.

Besides the skin, all the visible mucous membranes may be affected, but particularly those of the mouth, nose and pharynx. Lesions in the cloaca are mentioned. The eruptions on the mucous surfaces first appear as white, opaque, slightly elevated spots 1 or 2 mm. in diameter. The lesions rapidly increase in size, often coalesce, become ulcerated and covered with a white or yellowish, cheesy, necrotic material, which is probably the result of secondary infection with a variety of microörganisms. If the lesions be extensive the entire pharynx and even the larynx may become involved, and eventually filled with a membrane of microörganisms, necrotic material and exudate. The membrane may resemble that caused by the Klebs-Loeffler bacillus; and much effort has been expended in an attempt to establish the true relationship of fowl-pox to avian diphtheria. The mucosa of the nose becomes inflamed, and the air passages occluded by exudate, resulting in great difficulty in breathing. In fatal cases obstruction to air passages often seems to play an important part by interfering with breathing, and the terminal stages are frequently associated with a deep cyanosis. At autopsy one may find lesions on the mucosa of the pharynx, esophagus and crop similar to those in the mouth.

The general symptoms which accompany the eruption and become progressively worse as the disease develops, are loss of appetite, dullness, ruffling of the feathers, emaciation and pallor of the comb and wattles. An accompanying elevation of temperature is questionable. The cachexia increases and often becomes extreme. In fatal cases cyanosis appears and death occurs about 4 or 5 weeks after the beginning of the infection. Bollinger thinks the immediate cause of death in many cases is an interference with breathing. The mortality, according to Csokor, is dependent upon the situation of lesions in the throat. No doubt secondary invasion by bacteria and other microörganisms sometimes contributes to the fatal outcome (Reischauer). At autopsy no specific changes have been observed in any internal organ or tissue.

In the event of recovery the superficial lesions dry up and begin to fall off after the fourth or fifth week, and the animal begins to recover its appetite but may remain emaciated for several weeks, after which it gradually returns to a normal condition. Recovery from an attack is associated with an immunity.

Distribution of the Virus in the Infected Animal

The virus of fowl-pox is present in enormous quantity in the skin lesions. A slightly cloudy suspension of a nodule ground in saline solution is infective in a dilution of 1:2000 (Burnet). Consequently scrapings from the lesions or the dried crusts separating from them offer a rich and extensively used material for experimental inoculation. Material from early lesions of the mucosa are also highly infectious, but are not so suitable for an inoculum because of the abundance and variety of microörganisms, and the absence of desiccation *in situ*. Notwithstanding the negative results of Reischauer with blood and lymph, Loewenthal, Burnet, Lipschütz and Sanfelice fairly regularly found the blood to be infectious even after recovery from the disease. Sanfelice noted that the blood was most infectious at the height of development of the cutaneous lesions; thereafter it gradually became less virulent.

As might be expected, with virus circulating in the blood stream, many of the internal organs have been shown to contain the infectious agent. Loewenthal successfully inoculated pigeons with liver emulsion. The skin everywhere contains virus, and the brain and nerves were found to be infectious by Lipschütz. Sanfelice demonstrated virus in the spleen, lungs, liver and kidney, in the last two organs 24 hours after a successful inoculation upon the skin. It was likewise present in the internal organs 71 days after inoculation. The ovary was infectious in

the experience of Levaditi and Nicolau. It is thus evident, as Lipschütz points out, that fowl-pox must be considered a generalized infection and not merely a skin disease as was presumed by earlier writers. Probably all the organs and tissues harbor the virus, but only the cells which possess an especial affinity are able to localize it, so to speak, and to manifest a reaction to its presence.

The Inoculated Disease

The infectious nature of fowl-pox, long suspected, was proven experimentally by Bollinger, who succeeded in transmitting the disease both by contact and by inoculation. For the latter procedure he injected subcutaneously fragments of a spontaneous lesion. The experimental lesions were similar grossly and microscopically to the original ones. Since Bollinger's work the experimental production of the disease has been widely used in studies of the infection. Pigeons and hens have served almost exclusively for this purpose, and no essential differences have been observed in the disease in these two fowls, inasmuch as the results from the one group are applicable to the other. The pathogenicity of the viruses, however, is different, as mentioned above.

Experimentally, lesions of fowl-pox may be induced easily in susceptible fowls in a variety of ways, using as the inoculum material from the superficial lesions, blood or emulsions of various organs at the height of the disease. The most common practices are the rubbing of infectious material into slight injuries or scarifications of the skin, and intravenous injection. Light scarifications on the comb, wattles, eyelids or on the skin elsewhere, when inoculated by application of virus, show within 3 or 4 days light grayish lines outlining the scarifications which rapidly develop into grayish or yellowish, elevated, firm markings, becoming confluent when close together and presenting all the characteristics of a single nodule in a spontaneous eruption. If the feathers be plucked from any area of the skin and virus rubbed upon the small openings, nodular lesions, in every way identical with those of the spontaneous exanthem, will appear at the site of each feather follicle.

After an intraperitoneal injection, a specific nodule may develop at the site of needle puncture, but no characteristic lesions appear in the peritoneal cavity, nor does a cutaneous eruption ensue (Loewenthal). In pigeons Loewenthal repeatedly but not regularly induced typical exanthems by intracutaneous injections of blood and of emulsions of liver.

Although Loewenthal was unable to infect the cornea, this has been done quite successfully and regularly by Reischauer, Burnet and Lipschütz. Reischauer had better success in this respect with hens than with pigeons, and he states that corneal infection sometimes occurs in the spontaneous disease. The early lesions on the cornea are quite transparent and may escape notice. Later there is light clouding, and on healing a whitish spot may remain. Following the injection of masses of virus into the trachea Loewenthal discovered no essential changes either in the trachea or lungs. In the brain of certain chickens inoculated intracerebrally with fowl-pox virus Levaditi and Nicolau found lesions comparable to those of vaccinal encephalitis in the rabbit, characterized by perivascular infiltration, and parenchymatous alterations.

While inoculations induce typical nodules locally, more or less extensive depending on the extent of injury or scarification, ordinarily no secondary eruption develops elsewhere, although virus may be demonstrable in the blood stream and in the internal organs. However, the writer has frequently seen a secondary eruption about the head and on the buccal, nasal and pharyngeal membranes, usually limited in extent, but quite similar to the spontaneous lesions affecting these surfaces. This has been observed in hens inoculated upon a rather extensive area of the breast, especially if the local lesion after full development had been severely traumatized by scraping or partial excision. The secondary eruption has always occurred after the local lesions were well advanced.

According to Burnet the only experimental procedure which reproduces exactly the picture of the natural disease is intravenous inoculation. However, Loewenthal observed typical secondary eruptions after intracutaneous injections of emulsified liver. After an incubation of about 5 days, the nodules always appear at the points of election for the exanthem of the spontaneous disease, namely, about the head, the buccal mucosa, the tongue, eyelids and angles of the beak. If after intravenous inoculation feathers be plucked or if the skin be scarified, the specific lesions will develop at the sites of cutaneous injury. If one completely plucks the fowl it will become covered with specific nodules. Burnet thus sums up the requirements for the development of eruptive lesions: the virus in the circulation and a susceptible point. Manteufel, however, frequently observed both spontaneous and induced infections to proceed accompanied by general symptoms but without local lesions. These animals upon recovery were immune.

Pathological Anatomy

The eruptive nature of fowl-pox and the superficial resemblance of the exanthem to that of variola led earlier observers to the conclusion that the two diseases were the same. The true differences and similarities of the lesions in the two diseases, however, became more apparent as a result of accurate histological studies. The first careful observations of this kind were made by Bollinger who studied both spontaneous lesions and those induced by successful inoculation of the virus. His studies were made with the lesions from hens, though numerous subsequent studies have been made with nodules from pigeons, and the histology of the two has been found to be essentially the same; consequently no distinction will be drawn between the two species in this discussion of the pathological changes.

On microscopic examination Bollinger discovered that the lesions represent fundamentally a newgrowth of epithelial cells, differing in this respect from those of variola. There is no area of necrosis nor pustule in its center. He recognized a similarity of the lesions to those of molluscum contagiosum of man as described a few years before by Virchow; and he classified the lesion with tumors. Because the exanthem seemed quite distinct histologically from that of variola, and because he was unable successfully to inoculate a goat and a sheep with the hen virus he discarded the name fowl-pox and substituted epithelioma contagiosum to designate the disease.

Somewhat earlier Rivolta (1869) had studied material from the lesions microscopically and had found structures in the affected cells which he considered to be parasitic gregarines. Bollinger noted that the epithelial cells contained without exception a fairly large round or oval nucleus (Kern) which had a characteristic fat-like refractiveness, and filled almost half the cell body. No nucleoli were seen. He did not consider these to be inclusions or related to the etiology; on the contrary he was unable to discover, although especial attention was directed to this point, anything which with the slighest justification in his opinion might be considered to be the virus. Csokor identified epithelioma contagiosum of fowls with molluscum contagiosum of the human. He recognized the inclusion bodies within the epithelial cells of the lesion and thought them to be identical with those described by Henderson, Paterson and Virchow in the molluscum lesions. He speaks of them as molluscum bodies and was uncertain whether they could properly be considered to be independent microörganisms. Many features, however, suggested to him that they might be gregarines.

Benda who had previously studied the histology of molluscum contagiosum and had come to the conclusion that the molluscum bodies were not parasitic, found similar cellular inclusions in the epithelium of the nodules of contagious epithelioma, and considered them to be amebae. In discussing this paper Ziegler stated that he believed the inclusions of molluscum contagiosum to be parasitic, probably sporozoa, and not cellular degenerations.

Rivolta and Delprato (1880) after a very careful study of fowl-pox came to the

conclusion that the etiological agent was a parasitic plant, and because the microorganism (cellular inclusion) penetrated the epithelial cells, proliferated and increased in size there, they designated the supposed parasite *Epitheliomyces*, and the eruption an epitheliomycosis. These authors give an interesting description of the intracellular inclusions which are thought to be the microorganisms.

Mingazzini thought the cellular inclusions represented parasites, but believed the visible ones were mature and must pass through an intermediate host to become again infectious. Neisser regarded the inclusions as coccidia. Casagrandi isolated blastomycetes from the lesion. Pfeiffer (1889) ascribed the disease to parasitic flagellates.

After a thorough microscopic study of the lesions and of the epithelial inclusions, which he pictures accurately, Sanfelice attributed the infection to blastomycetes which he cultured; and with them he claimed to have reproduced the disease. In his histological description he found the smallest intracellular inclusions in the deeper Malpighian layers. These bodies increased in size within the cells outward to the surface.

Polowinkin (1901) was among the first to sound a discordant note in the interpretation of the intracellular bodies of fowl-pox. He observed that the lesions consist of an epithelial hyperplasia, and also noted an inflammatory infiltration of the corium and underlying connective tissue. The inclusions, which other authors had interpreted as gregarines, coccidia, amebae and blastomycetes, he maintained were nothing more than products of degenerative processes within the cell. He showed that the tumors induced by inoculating blastomycetes consisted of granulation tissue and were not, as maintained by Sanfelice, related to the specific nodules of fowl-pox. On the other hand he found a small rod-like microorganism in the blood and organs of infected pigeons which he was inclined to regard as the true etiological agent.

Up to this time little attention had been paid to histological and cytological minutiae. It was generally recognized that the eruptive lesion of fowl-pox was essentially an epithelial growth, associated sometimes with inflammatory infiltration of the underlying tissues. These were the days when the search was on for protozoan and plant parasites as etiological factors of disease, and attention was particularly focused upon the cellular inclusions which Rivolta had seen. These indeed were the most striking feature of the lesion. They were found to be conspicuous round, oval, club-like or mulberry-formed, sharply defined, refractive, sometimes coarsely granular masses which occupied the cytoplasm of almost every epithelial cell of the lesion. In size they varied, being smaller in the deeper layers of the Malpighian cells, becoming larger toward the periphery. In the deeper cells there were often several small bodies but further out only a single large body lay in the cytoplasm, usually almost filling it, but still surrounded by a zone of cytoplasm. The nuclei of the affected cells were more or less intact. The bodies, in fact, did not resemble any derivative of a cell hitherto observed save the bodies of the similar infectious disease molluscum contagiosum of man. The almost universal interpretation, therefore, was that they represented independent microörganisms, protozoan or plant, which had penetrated the cells and were growing in symbiotic relation with them.

At this juncture a new fact was discovered which profoundly influenced future

cytologic and etiologic investigations. In 1902 Marx and Sticker succeeded in filtering the virus through a Berkefeld filter; and they induced the disease with the filtrate. They, therefore, disregarded the previously described etiological agents and definitely placed the virus among the filter-passers. They further emphasized certain characteristics of the virus, namely, that it withstands complete drying and exposure for many weeks to diffuse daylight and sunlight. It is apparently unaffected by a temperature of −12°C. It withstands 60°C. for 3 hours, and 100°C. for 1 hour, if previously dried and sealed in vacuum tubes. It survives many weeks in glycerol; and it induces immunity.

This was a blow to the older theories and necessitated further investigations and new conceptions, for obviously the inclusions under consideration were too large to be considered seriously as filter-passing agents. Cytological studies were now concentrated on the origin and intimate structure of the inclusions; and the adherents of the protozoan hypothesis were forced to construct complicated life cycles, one phase of which was filter-passing.

In 1903, Michaelis reported an interesting microscopic study in which he called attention to the fact that the specific inclusions of fowl-pox give the reactions for fat, which was first noted by Benda (unpublished work). The substance reacted positively to Scharlach R and to osmic acid. He found that two constituents could be demonstrated microscopically; the fatty material, first appearing in tiny globules in the cell and finally coalescing, and an albuminoid material which could be stained after removing the fatty substance with alcohol. The fatty material had no structural peculiarities. With the mordanting method he distinguished within the bodies a strongly stained outer zone or ring and a clear unstained central portion. Sometimes the inclusions presented a mulberry appearance. He regarded the inclusions as typical of fowl-pox, but did not pass judgment on their nature, considering both the possibility of a parasitic and a degenerative origin. In the latter event they probably had to do with albuminous keratinization. No structures in the normal cell were found which gave rise both to the fat and the mordant reactions.

About the same time, Apolant, also mindful of the filterability of the virus, published a cytologic study in which he went into great detail as to the origin and significance not only of the larger characteristic structures in the cell but of certain smaller inclusions which he called Benda bodies. These were analogous to similar structures described by Benda in the cells of molluscum contagiosum, and considered by him to be the virus, while the true molluscum bodies were viewed as degenerations. Apolant decided that the Benda bodies are particles extruded from the nucleus. They occur usually about the nucleus; they are often surrounded by a halo, and eventually undergo dissolution. The fowl-pox inclusions proper, he found, arise entirely within the cytoplasm. It seemed doubtful to him that fat is an essential constituent of these inclusions inasmuch as it varies in amount within wide limits, and sometimes is almost completely absent. He concluded that the Benda bodies arise from a degeneration of the nucleus, the characteristic fowl-pox bodies through a degeneration of the cytoplasm, there being no direct relation between the two except that they are both the expression of the activity of the same virus.

Neither of these contributions offered anything relative to the nature of the virus. Morphological investigations, however, did not long remain unfruitful,

and in 1904 the illuminating observations of Borrel not only gave an added impetus to these studies but founded a new conception of the structure of the viruses of several diseases (contagious epithelioma, molluscum contagiosum, sheep-pox, vaccinia) in harmony with the newly discovered fact of filterability. In smear preparations from the cutaneous lesions of fowl-pox, stained by Loeffler's flagella method, Borrel discovered myriads of minute structures, micrococcal, isolated, in diplococcal, staphylococcal and chain forms. About each element appeared a well-defined sort of mucous capsule. Here was something which had all the appearances of a very minute microörganism. It was present only in the specific lesions and in enormous numbers, sufficient in fact to account for a virulence in high dilution of the infectious material. Further it appeared small enough to make obvious the possibility that it might traverse the pores of a Berkefeld filter, as is stated to be the case (da Rocha-Lima). This fact was new and of the greatest importance. Borrel's discovery naturally suggested that the diminutive bodies might be minute cocci and the specific etiological agent of the disease.

Not long afterward Lipschütz demonstrated similar minute structures in the closely allied disease molluscum contagiosum, and Paschen analogous bodies in variola and vaccinia. But Borrel could not discover the minute bodies, which were abundant in smears, in sections of fixed tissue, and he was uncertain as to their relation to the specific inclusions.

In a very thorough study of fowl-pox, Burnet completely confirmed Borrel's observations. In a careful analysis of the pathology of the lesion, he stated that wherever the inclusions are found there the virus is also. Three hypotheses were proposed as possible explanations of the nature of these specific inclusions, namely, (1) they are parasitic; (2) they are the products of secretion or degeneration of the cell, brought about through the action of the unknown virus; (3) the inclusions envelop in a substance of cellular secretion or degeneration a microorganism, whose form accords with the acquired facts in regard to abundance and filterability of the virus, a theory which Volpino had proposed for the Negri bodies. Burnet judged from stained preparations that the inclusions and the granular masses seen in fresh preparations, which Borrel showed could be broken up into myriads of minute coccus-like bodies, were derived from the same cell. The discovered facts, he believed, imposed the hypothesis of a minute microorganism, in intimate association with the specific inclusions, as the etiological agent.

He points out that if one takes into consideration the apparent enlargement due to the staining, the bodies seem to be of about the order of magnitude of the microbe of peripneumonia, a filter-passing agent cultivated by Nocard and Roux. They are much smaller than many bacteria which passed the filters used in his own experiments. To the hypothesis of a micrococcus he added that of a zoöglia, giving the appearance of the inclusions in sections. The enormous number of the micrococci accords in his opinion with the extraordinary abundance of the virus. Their immobility and the sheath which surrounds them explain the difficulty with which they are filtered. The masses of virus contained within the inclusions might explain the unusual resistance of the infectious agent.

Borrel's discovery had further confirmation in the experience of Lipschütz and others, though Loewenthal still regarded typical inclusions of this and other similar diseases as the product of cellular reactions dependent on the one hand upon the kind of disease, and on the other upon the type of cell affected.

Lipschütz succeeded in staining the Borrel bodies not only by Loeffler's method, but by Giemsa's stain. He explained the inclusions as the result of degenerative changes in the cytoplasm called forth by the irritation of the intracellular parasites which penetrate the cell. Several years later, however, following the demonstration by da Rocha-Lima, that, if the inclusions of fowl-pox be suitably extracted with lipoid solvents, they can be seen to be composed of minute granules, he changed his view of their nature, conceiving of them as intracellular colonies of the virus.

Disregarding Borrel's discovery or unaware of it, Reischauer described in great detail complicated structures which he found in the affected epithelium, in the connective tissue, between cells and in the blood of infected fowls and which he believed to be the virus. He considered them analogous to the inclusions found by others in variola and vaccinia. He believed that a very close relationship, if not an identity, exists between the viruses of various types of animal pox.

Sanfelice, having abandoned his earlier view that the infection is due to blastomycetes, completely discarded the theory of a parasitic origin of the disease and submitted experiments designed to show that it is the effect of a non-living toxic substance which, through the cellular injury it induces, is capable of reproducing itself. The inclusions he now held to be the products of cellular degeneration, derived essentially from nuclear extrusions.

Recently (1926) in a cytological reinvestigation of the lesions, Ludford and Findlay also accept the intracellular bodies as degenerations. They add the information, however, that mitochondria do not appear to be concerned in the formation of the virus bodies. The latter are in many cases in close relationship with the Golgi apparatus, which undergoes a complete reversal in polarity in affected cells. No mention is made in their paper of the work of Borrel, Burnet, v. Prowazek, da Rocha-Lima or Lipschütz.

The minute bodies composing the intracellular inclusions of molluscum contagiosum and of fowl-pox, and similar bodies which Paschen has shown to be so characteristic a feature of the lesions of vaccinia, are not to be thus abruptly disregarded, in the writer's opinion, as etiological possibilities. The fact that no one has as yet succeeded in cultivating these bodies leaves them for that matter in a plight not much worse, etiologically considered, than that of the acid-fast Hansen bodies of leprosy. Evidence for or against their origin from degenerative or reactive processes within the cell is, however, to the point.

In an attempt to determine whether or not the specific intracellular bodies of fowl-pox are derived from any demonstrable cellular constituent the writer has made a study of their origin and mode of development within the cell.

The virus used was a strain derived from a case of spontaneous fowl-pox sent to us by Dr. Beaudette of the New Jersey Agricultural Experiment Station. When inoculated on the scarified skin of a susceptible chicken a lesion develops to its height on about the sixth day. Pieces of tissue excised from the developing

lesion have been the material studied. As with molluscum contagiosum the best fixative for the study of the affected epithelial cells was found to be Regaud's, 24 hours, followed by a mordanting in 3 per cent potassium bichromate for 3 to 4 days. After this fixation the intracellular virus bodies are differentially stained a deep blue with Mallory's phosphotungstic acid hematoxylin stain. For the demonstration of mitochondria the acid fuchsin methyl green method has been employed.

The changes in the epithelial cells develop very similarly to those which take place in the lesion of molluscum contagiosum. The well-developed lesion consists essentially of an epithelial hyperplasia involving both epidermis and underlying feather follicles. There is more cellular reaction in the subcutaneous tissue than is found in molluscum contagiosum. The basal layer of epithelium shows little change from the normal. Peripheral to the basal layer the earliest stages in the development of virus bodies appear. The cells in which they occur are enlarged and the cytoplasm is abundant. The mitochondria are thread-like and larger than in the cells of the basal layer. The material constituting the virus body appears first about the periphery of one or more vacuoles commonly situated at the proximal pole of the cell. In suitably stained preparations it has the appearance of an amorphous granular substance, and may be stained lightly with osmic acid. One or several vacuoles may occur in the same cell. Nearer the surface, the altered cells become yet larger and there is evidence of a change in position of the virus bodies, so that they come to lie more frequently at the distal pole of the cell. Extruded nuclear particles appear within the cytoplasm, and they can be sharply distinguished morphologically and tinctorially from the virus bodies. They are spherical, sharply outlined masses without a central vacuolation, possessing a basophilic reaction, so that they stain green in contrast to the deep red of the virus bodies, in acid fuchsin methyl green preparations. The mitochondria become fragmented and granular, and it appears that particles of their substance may adhere to the virus body.

The virus body increases rapidly in size. Its outline in fixed preparations becomes irregular and coarsely granular obscuring the original vacuole. As the surface of the lesion is approached the cells show evidences of disintegration. The mitochondria become granular and vesicular, the nuclei shrunken and irregular in their staining reactions. The virus body may become more or less isolated within the cytoplasm by fluid, as if the cytoplasm about it were very edematous. On the surface there are great alterations of the cells from desiccation, the presence of bacteria and other external influences.

Throughout its residence within the living cell the virus body increases in size until its volume may become as great as that of the original cell. Its enlargement is due to a generation of its essential substances, and there is no evidence that these substances are derived from any demonstrable cellular constituent, except perhaps a small amount of mitochondrial material which may become adherent and possibly incorporated with it.

If the surface of such a lesion be slightly scraped and the scrapings moistened with water and pressed under a coverglass, the virus bodies may be easily recognized under the microscope. When they are pressed out thin beneath the coverglass they are seen to be composed of myriads of minute bodies which seem to be agglutinated by a viscous material between them. In smears stained with Loeffler's flagella stain, or better with carbol-anilin-fuchsin for 1 minute, after mordanting an equal period with $\frac{1}{4}$ per cent potassium permanganate (Goodpasture), they are readily demonstrable. They are round or slightly oval, sometimes arranged in short chains or in diplococcal and biscuit forms. They are colored a distinct pink by the latter method, and are Gram-negative. These bodies may be equally well studied in dark-field preparations. They measure about 0.25 μ in diameter, and have the appearance generally of a minute, non-motile microörganism.

The ease with which they may be demonstrated in smears stained by the carbol-anilin-fuchsin method makes it a simple matter to follow them in various culture media. The striking morphological similarity to a living organism and the readiness with which they may be demonstrated should make them serve as a logical point of departure for cultural investigations, and studies of their reactions under various circumstances.

In unpublished work in the writer's laboratory, in conjunction with R. C. Avery, the observations of Borrel, Burnet, Lipschütz, da Rocha-Lima and others regarding the presence of the coccus-like bodies in the lesion have been completely confirmed. It has further been possible definitely to associate them with the specific cellular inclusions. Briefly the inclusions examined in fresh preparations are found to contract in hypertonic salt solutions and to swell very greatly when placed in distilled water. In the swollen state they appear to be composed of several coherent globules of fluid held together apparently by a semipermeable albuminous material. Within the largest globules the minute bodies are to be clearly seen dancing in an exceedingly rapid Brownian motion. On the readdition of salt solution the inclusions rapidly contract be-

coming again hyaline. When compressed the mass ruptures, still remains coherent and is uniformly finely granular. In the fresh preparations the uniform minute bodies which have become extruded are very easily seen. On staining such a preparation the compressed inclusions are found to be composed of the Borrel bodies mantled by a gelatinous material in which they lie; and their staining property seems to be totally or partially dependent upon the envelop of his material which adheres to them.

It has likewise been shown that these minute structures are easily demonstrable in the dried scabs 6 months after desiccation, by soaking the scab in distilled water 24 hours and making smears. Likewise they are found after preservation in glycerol. On the other hand, under conditions which destroy the virus, such as incubation at 37°C. in saline solution for 10 days or 2 weeks, the addition of KOH, prolonged putrefaction, the bodies disappear apparently coincidently with the virus. It is possible to procure a fairly pure suspension of the unruptured inclusions, for the most part free of cells. After centrifuging and washing several times in physiological salt solution, the supernatant fluid proved to be entirely innocuous, while the sediment was highly virulent. Thus the virus is associated with the inclusions, as Remlinger many years ago demonstrated to be the case with the sediment of rabic cord.

At least from a morphological standpoint a thoroughly logical hypothesis has been evolved which seems to harmonize with the known facts about the virus of fowl-pox. This hypothesis interprets the specific cellular inclusions to be aggregations or colonies of a tiny coccoid microorganism held together in a zoöglial mass by an albuminous material which is usually associated with a lipoidal constituent. The evidence seems to the writer preponderant that the minute bodies, which are the essential components of the intracytoplasmic structures peculiar to the lesions of contagious epithelioma, are in reality living agents.

The Virus

The epithelial lesions of fowl-pox are enormously rich in virus, and this material has been largely used in experiments to determine its various properties. Highly infectious material has been subjected to a great number of experimental procedures, the results of which may be catalogued briefly.

Filterability.—While today the filterability of a virus perhaps does not connote the same degree of exoticism in the infectious agent, such as ultravisibility or fluidity, as it may formerly have done, yet the

demonstration that the virus of fowl-pox can pass the pores of a Berkefeld filter (Marx and Sticker) marked an epoch in etiological investigations of the disease. Although Marx and Sticker succeeded with a Berkefeld filter, experiments with the porcelain filter (Chamberland F) were negative. Their results have been confirmed by Juliusberg and by Burnet. The latter worker observed that the virus passed the filter with less frequency and abundance than did the motile cholera vibrio. It was exceptional in his experience that it filtered without the passage at the same time of the cholera vibrio, or the non-motile bacterium of chicken cholera. There seemed to him no reason for assuming the existence of an invisible, or for that matter an excessively small microorganism, as the causative agent, at least not necessarily below the order of magnitude of the germ of peripneumonia.

A delayed incubation period following inoculation with filtrates was noted by Marx and Sticker and by Juliusberg. The time before the appearance of lesions was prolonged to about three times that observed on inoculation with unfiltered material. The view of the latter author that this delay is probably due to a necessary growth process of the filtered portion of the virus before it becomes infectious, seems less acceptable than the explanation suggested by experiments of Sanfelice, who found that if virus were sparsely present in the organs of an infected pigeon the incubation period of the resulting inoculations was similarly prolonged, as compared with inoculations with organs rich in virus. In other words it seems to be the relatively small quantity of virus in the filtrates, rather than any inherent characteristics of the virus, which is responsible for the delayed appearance of the lesions.

A distinction is to be noted between the virus of epithelioma contagiosum of fowls and that of molluscum contagiosum, in that the latter will pass through a Chamberland filter (Juliusberg).

Biological Variations.—There is an evident biological difference between the strain of virus adapted to hens and that indigenous to pigeons. The pigeon virus is inoculable upon hens, but it is rare, if ever, that the hen virus will infect pigeons. The efforts of Marx and Sticker in this direction were entirely negative. The pigeon virus indeed may be attenuated for the same species by a single passage through the hen. Thus, in the experiments of these authors, 18 pigeons were inoculated with virus from hens infected with pigeon virus. In 3 the disease was quite abortive, in 5 so mild that the lids were not closed, in 3 the disease was quite typical, in 7 no infection resulted. In all, the incubation period was considerably prolonged (9 or 10 as against 3 or 4 days). Marx and

Sticker state that the virus of hens is also easily attenuated by continuous passage in hens. Juliusberg observed a diminution in activity to complete avirulence of pigeon virus by repeated passage in the pigeon. Burnet on the contrary saw no change in the virulence of pigeon virus with a considerable number of passages during a period of more than a year. The experience of Sanfelice was the same.

Pathogenicity for Mammals.—The virus of contagious epithelioma is not infectious for man (Pfeiffer, Juliusberg), for goats and sheep (Bollinger), and for mice (Juliusberg). Its pathogenicity for the rabbit seems questionable. Levaditi and Nicolau state that the virus has a feeble affinity for rabbit skin. They observed papular lesions on the shaved and scarified skin following inoculation. These lesions were characterized histologically by perivascular infiltration and epithelial proliferation. No mention is made of specific cellular inclusions. Andervont concludes that the virus of epithelioma contagiosum is mildly pathogenic for rabbits, although it does not induce lesions resembling those of the chicken. His only evidence of pathogenicity seems to be the fact that virus was demonstrated 4 days on the scarified skin and 3 days on the scarified cornea, while similarly inoculated unscarified tissue was not infectious after these periods. The virus was lost in attempted rabbit to rabbit passage. More significant are the recent results of Lusena who succeeded in establishing the virus in the body of rabbits by cutaneous and by intratesticular inoculations. His experiments were performed with a strain (van Heelsbergen) of pigeon virus. Rabbits inoculated with the virus showed at no time clinical symptoms or histologically recognizable changes. However, following cutaneous or intratesticular inoculation the virus became generalized in the rabbit as in the fowl, and remained active for from 24 hours up to 65 days in its blood, tissues and organs. Lusena thinks that the virus increases actively in the rabbit's body and possesses an especial affinity for the skin. The virus through repeated rabbit passage may in certain cases be heightened in virulence. The passage virus continues to induce in pigeons lesions similar in histological character to the original ones. Pregnant infected rabbits remain sound, but the blood and tissues of their young become infected with the virus. This appears to be a curiously passive infestation and well worth further study in the rabbit and if possible in other animals.

Affinity for Tissues.—Practically all authors agree that the virus has an especial affinity for squamous epithelial cells of the skin and the outer mucous membranes, and that it is present in highest concentration

within and about these cells of the lesion (Burnet, Lipschütz, Sanfelice). Ledingham, on the contrary, is of the opinion that the reticulo-endothelium is primarily attacked. He regards the inclusions of fowl-pox as foci of keratinization within the cell. The underlying vascular changes are to his mind unmistakably analogous to those met with in vaccinia, in which disease he finds no affinity of the virus for epiblastic elements, but a primary attack on the reticulo-endothelium.

It is certain, however, that squamous epithelium is profoundly affected by the virus, even before any significant changes are to be found in the underlying tissue. It was observed by Burnet that the subcutaneous reaction is more marked in the case of a benign eruption, attenuated or abortive, than in a more intense eruption of long duration. As a result of histological studies of various stages of the infection in the same fowls, it is the opinion of the writer that the subcutaneous inflammatory reaction is a manifestation of a defensive response, gradual at first and increasing with the duration of the disease in proportion to the amount of virus and to the length of time the virus present in the overlying epithelium is directly exposed to the reacting corium and subcutaneous tissues. It seems to correspond to the development of immunity.

Cellular inclusions have not been observed in cylindrical epithelium, or in any internal organ. As has been stated, however, there is practically a universal dissemination of virus through the blood stream. The bodies of Borrel have not been found either in the blood or in the internal organs.

It seems very doubtful that the virus, in spite of its wide dissemination, attacks specifically any tissue save epithelium at the site of the eruptive lesions. The one great factor determining the localization of the virus with the resultant formation of a lesion is epithelial injury, whether the virus is applied externally or whether it already circulates in the blood stream. It has not been determined whether the injury exposes specifically susceptible cells to penetration by the virus, or stimulates the production of young immature cells which are especially sensitive to its influence. A second fact of importance is that the virus increases enormously locally within the lesion. That it can similarly increase actively within the blood stream or internal structures is doubtful; at least the occurrence of virus within the interior of the body may be explained on the basis of a more or less passive spread from a local lesion to circulate until again localized at certain sites of predilection or through injury. Experiments of Sanfelice bear on this point. He found that the increase of virus in internal organs coincides with the extent of

the skin lesions, diminishing with their healing; and he interprets this fact to mean that the skin lesions are really the focus of proliferation of the virus. With the well-established fact of high resistance which the virus enjoys and the absence in the blood of demonstrable virucidal substances, it is perhaps not surprising that it may circulate in the blood or lodge in internal organs and persist there quite inactively.

Resistance of the Virus.—One of the most characteristic properties of the virus is its high degree of resistance to desiccation (Marx and Sticker). Material from lesions dried and kept for 2 years at room temperature has proven to be infectious (Loewenthal). Desiccation either gradually at room temperature or rapidly over sulphuric acid is a good means of preserving the virus. A fine suspension of virulent material spread upon glass slides and dried slowly in the air was infectious after 15 months (Burnet). Resistance to drying is no doubt an important factor in the persistence of the virus under natural conditions, and Marx and Sticker. have demonstrated that it will also withstand exposure to diffuse daylight and sunlight for many weeks. Burnet found that scabs exposed to light under glass (Petri dish) were still infectious after 2 months. A mixture of a 1 per cent solution of erythrosin and a like amount of filtered virus is inactive after exposure to daylight for 3 days (Juliusberg). The virus, suspended in water kept in a refrigerator where the temperature does not go below 6°C., will survive for a surprisingly long time. Under these conditions, according to Burnet, it was still active after 60 days, and after 30 days at 22°C. It survives –12°C. (Marx and Sticker).

Thus the virus is eminently adapted to a survival for long periods when exposed to out-of-door conditions, such as dryness, moisture and light, especially during cold seasons; and the possibility of its survival through the winter in the barnyard, as indicated in the observations of Bollinger, seems obvious.

Moist heat destroys the virus fairly readily. Burnet observed it to be active after 3 days, but inactive after 8 days, when suspended in water in an oven at 37°C. A fine suspension in physiological saline solution or tap water in sealed ampoules was inactivated in about 8 minutes at 60°C., but nodules and scabs similarly treated contained active virus after exposure for 1½ hours. At 56°C. the virus in fine suspension did not survive more than ½ hour, but a fragment of the lesion forced into gelatin still induced the disease after 14 days at 38°C.

Marx and Sticker found the virus to be active after 3 hours at 60°C., but in the opinion of Burnet the suspension could not have been very fine. The same authors demonstrated active virus in pieces of nodules

which were desiccated, sealed in ampoules deprived of air, and heated for 1 hour at 100°C. According to Reischauer the virus resists dry heat 15 to 30 minutes at 80°C., and moist heat 5 minutes at 100°C. Loewenthal exposed virus to radium emanations and found it active after $5\frac{1}{2}$ hours. Preserved in glycerol virus was active after 120 days (Burnet), and after many weeks (Marx and Sticker).

The virus has been exposed to a variety of chemicals with the following results. In an emulsion of scabs, it resisted 1 per cent phenol water $1\frac{1}{2}$ hours; 2 per cent inactivated it in 30 minutes (Marx and Sticker). It is inactivated in 5 minutes by 1 per cent KOH, 1 per cent acetic acid and bichloride of mercury 1:1000.

Sanfelice denies that it is killed by exposure to 1 per cent KOH for 24 hours, claiming that it is only inactivated and can be rendered active again by acidification. Lipschütz saw no change in the virus after an exposure of 2 hours at room temperature to 1 per cent atoxyl; 1 per cent saponin had no effect in 1 hour, but after 24 hours there was apparently some diminution in infectiousness; 10 per cent sodium taurocholate gave variable results, the virus in very dilute suspension was destroyed on one occasion in 2 hours. The same author observed no effect of salvarsan upon it either *in vivo* or *in vitro*. Manteufel noted that while bile does not destroy the virus, it decreases its virulence.

Cultivation Experiments.—All attempts thus far to cultivate the virus of fowl-pox have been unsuccessful. Microörganisms grown from time to time have been discarded in the light of further experience. In 1910 Bordet and Fally reported the successful cultivation on artificial media (the Bordet-Gengou medium for *B. pertussis*) of the etiological agent of avian diphtheria. They carried the microörganisms in pure culture through many generations and reproduced the disease with the cultivated organisms. The microörganism was extremely small, stuck together in zoöglial masses and was difficult to stain.

Previous attempts had been made to prove the identity of avian diphtheria and epithelioma contagiosum, but contrary to the opinion of Carnwath and of Schmid, Bordet and Fally expressed the belief that the two diseases have not a common etiology. Notwithstanding the position of the latter workers on the question, Lipschütz regards the evidence as conclusive that the two diseases are identical, implying that the microörganism cultivated by them is to be regarded as the etiological agent of both, though he himself was unable to cultivate it from typical lesions of fowl-pox.

The Relation of Epithelioma Contagiosum to Avian Diphtheria

It has been thoroughly demonstrated from studies of the spontaneous as well as of the inoculated disease, that specific eruptive lesions of the oral, nasal, pharyngeal and laryngeal mucous membranes are common accompaniments of the cutaneous eruption of epithelioma contagiosum. This has been noted by Bollinger, Pfeiffer, Friedberger and Fröhner, Reischauer, Burnet, Lipschütz and others. The observation has often been made that these lesions may spread and become covered by a membrane, being necessarily infected with mouth organisms, to such an extent that interference with breathing endangers life or actually proves fatal. Csokor thought the high mortality which sometimes attends the infection (20 per cent) is not due to the symptoms of the disease but to the situation of lesions in the mouth and pharynx bringing about a croupous or diphtheritic inflammation, which kills the animal. The severity and extent of the mucosal lesions vary greatly, and may partially depend on the types of microörganisms secondarily infecting them.

On the other hand, epidemics of a diphtheritic inflammation of the oral and respiratory mucous membranes occur, in which there are no instances of an accompanying cutaneous eruption. The question arises as to whether this disease, localized strictly in the respiratory mucosa, is always a manifestation of an infection by the virus of epithelioma contagiosum, or whether it may be due to one or more etiologic agencies of a different nature.

The evidence indicates that an epidemic due primarily to the virus of fowl-pox may exhibit its lesions entirely in the mouth and respiratory mucous membranes, exhibiting a diphtheritic inflammation. Such an inflammation was studied by Carnwath. Clinically this disease was avian diphtheria. In only one case was there a skin lesion, a small nodule situated on a wattle. Inoculations upon the comb were made with the diphtheritic membrane, and lesions, said to be typical of fowl-pox, resulted. No histological study was carried out. As a result of this study Carnwath thought it doubtful that a pure, primary bacterial avian diphtheria exists. He could not reproduce the typical disease with cultures of a variety of bacteria derived from the lesions. In conclusion he recommended, in order to prove his contention that fowl-pox is always the cause of avian diphtheria, the inoculation upon the skin of diphtheritic membrane from other epidemics to determine the presence of fowl-pox virus.

Shortly afterward this experiment was carried out by Schmid with 10

cases of avian diphtheria, the fowls coming from different places. In only 3 cases of the 10 did he succeed in inducing typical pox on the skin. With material from the remaining 7 he either induced more or less pronounced lesions of the mucous membranes, or the results were completely negative. In further cases Uhlenhuth and Manteufel were able to establish fowl-pox virus as the cause of diphtheritic inflammation in fowls. They recognized, however, that other agents might cause the clinical picture of avian diphtheria.

This seems to be the case, in view of the success of various workers in inducing diphtheria in fowls with pure cultures of bacteria derived from certain epidemics of typical avian diphtheria. Loeffler isolated *B. diphtheriae avium*, which Babes and Puscariu accepted as an etiological agent of the disease. Loir and Ducloux isolated a Gram-negative bacillus in pure culture from the spleen of chickens dead of diphtheria, with which they easily reproduced the disease. And it does not seem probable that their cultures were mixed with the virus of fowl-pox, inasmuch as the diphtheria was readily transmitted by contact, no local lesion developed in the skin at the site of subcutaneous injection, and artificial immunity could be established with attenuated cultures. Streit studied avain diphtheria extensively, without seeing among his cases a single instance of fowl-pox. He isolated two pathogenic bacteria from the lesions which were able to induce a diphtheritic inflammation on inoculation of pure cultures, namely *B. pyocyaneus* and a bacterium he calls the "Roup bacillus." He did not consider avian diphtheria an etiologically distinct disease. Fally could not demonstrate fowl-pox virus in the cases of avian diphtheria under his observation, and Bordet and Fally state that the diphtheritic disease from which they obtained their cultures of a specific microörganism was distinct from epithelioma contagiosum. Might not the virus which they grew, nevertheless, be the etiologic agent of fowl-pox? The zoöglial masses, the minute size, the staining characteristics, resemble indeed the Borrel bodies which may be demonstrated in the lesions of epithelioma contagiosum. However, no one has as yet been able to cultivate a similar microörganism from typical skin lesions of fowl-pox, using the technique of Bordet and Fally (Uhlenhuth and Manteufel, Lipschütz). In experimental inoculations through various channels with cultures of their virus Bordet and Fally never observed cutaneous lesions resembling those of fowl-pox, whereas the virus of fowl-pox injected intravenously usually results in a cutaneous eruption. On subcutaneous injection no local lesion seems to have occurred, nor did this method induce avian

diphtheria. A characteristic diphtheritic lesion could be induced with the cultivated organism when it was inoculated on the mucous membranes; even on the nictitating membrane a typical diphtheritic exudate with abundant purulent secretion developed within a few days. Fowl-pox virus from skin lesions inoculated upon the nictitating membrane does not of itself, in our experience, readily lead to a diphtheritic inflammation. Fally states that the recovery from an attack of avian diphtheria confers no immunity against contagious epithelioma and *vice versa*. Also in contrast to contagious epithelioma the blood from a case of avian diphtheria is not infectious.

With the evidence at hand we must accept the judgment of Bordet and Fally that the type of avian diphtheria which they investigated was not identical with epithelioma contagiosum, and as a corollary, that avian diphtheria is not an etiologically distinct and uniform disease. The artificially cultured specific agent of fowl-pox should be able to reproduce the cutaneous lesions of this disease, or at least to induce the specific cellular changes in the mucous membranes if not in the skin.

It may well be questioned, however, whether microörganisms which cause an injury to and incite an inflammation of the oral, nasal, laryngeal and pharyngeal mucous membranes may not frequently serve as agencies for the secondary establishment of an infection by fowl-pox virus upon the sites of injury thus prepared. Such an association of fowl-pox with a virulent bacterium might account for the rapid and thorough infestation of a flock of fowls, which under laboratory conditions are extremely resistant to contact infection when intimately exposed to animals bearing extensive lesions of the inoculated disease.

Immunity

It seems very probable that a natural immunity exists in certain individuals, both pigeons and chickens, to their respective strains of virus. Loewenthal encountered, though rarely, naturally immune pigeons. Burnet found none completely resistant but noted a difference in the degree of susceptibility, some easily becoming strongly immunized, others less so. The writer has observed chickens refractory to fowl-pox which had not apparently been exposed previously to the virus.

Fowls recovering from a severe attack of the disease possess an acquired immunity (Marx and Sticker, Reischauer), which according to Loewenthal, in pigeons at least, is strong but of short duration (2 months). Burnet did not observe such a prompt cessation of immunity. Numbers of his pigeons which had recovered 4 and 5 months previously

proved to be completely insusceptible to reinoculation. He made the important observation that there are degrees of immunity. After a very extensive eruption it is strong and of long duration, but if the lesions be circumscribed and benign the consequent immunity may be partial and very brief. Loewenthal attempted to determine the time of onset of immunity by inoculating several pigeons on the breast, and, at varying intervals, thereafter, excising the lesion and testing the immunity by reinoculation upon the eyelid. He found that the secondary infection showed no diminution in intensity up to the time when the lesion on the breast was so far advanced that the scab fell off. It seems evident that the inoculations upon the breast must have been small.

Burnet recognized the differences in degree of immunity resulting from variations in the extent of the disease, and estimated the period of onset of acquired resistance by inoculating pigeons on the breast and subsequently reinoculating them at varying intervals. Up to 5 days the response to reinoculation was equivalent to the control. The lesions of subsequent reinoculations gradually diminished in extent, until those made on the twentieth day completely aborted. The lesions of reinoculations healed contemporaneously with the primary lesions.

Loewenthal noted that immunity occurs in such a manner that a reinoculation presents, after an abnormally short incubation period of 1 to 2 days, a swelling suspicious of pox. This, however, disappears in 1 to 2 days more. A similarly rapid reaction was noted by Burnet and by Manteufel in partially immunized animals.

Intraperitoneal injection of virus may lead to a complete immunity or to a diminution in the severity of a subsequent infection (Loewenthal). Subcutaneous injections of 1 cc. of a tolerably thick suspension of virus, in the experience of Lipschütz, immunized some, but not all, pigeons.

Inoculations upon the cornea are of particular interest. Burnet found that the disease could be successfully inoculated upon the scarified cornea, the resulting lesions containing the specific cellular inclusions. A successful inoculation of a single cornea led to a complete immunity which appeared 17 to 19 days later. In repeating these experiments Lipschütz noted no cutaneous immunity up to 10 days; later some animals were immune, others not. He observed, however, that with primary cutaneous inoculation and subsequent inoculation of the cornea, in no instance, whether the skin lesion had been healed 2 months or was still present, could an immunity of the cornea be demonstrated. In this connection he calls attention to the observations of v. Prowazek, and of Kraus and Volk, that the cornea of a monkey (*Macacus*) whose

skin is immune to vaccine virus, remains susceptible to reinoculation, and that inoculation of the cornea leads to no cutaneous immunity. In view of later work, however, with vaccine virus and herpes virus in rabbits, it seems unlikely that so strict a local immunity exists as that suggested by these early observations. If one waits long enough before reinoculation the local character of the immunity is not so obvious.

Virus killed by heat and given either by way of the skin or intravenously (Burnet) does not immunize. How long an immunity will last is undetermined, but it has been observed to be complete up to a period of 6 months (Lipschütz), and 2 years (Manteufel).

Notwithstanding the existence of a complete immunity to experimental reinfection no virucidal effect of the serum has been demonstrated either in animals recovered from the disease or in those hyperimmunized (Burnet, Lipschütz, Sanfelice, Manteufel). Manteufel believes that the immunity is a general one and is not confined to particular tissues. Some fowls, following an intravenous or subcutaneous injection of active virus, may become infected and present the general symptoms, without local lesions. These fowls, upon recovery, are immune to inoculations upon the skin, and present no general symptoms following intravenous administration of the virus. The skin immunity, he believes, is not histogenous, but a sign of a general immunity. Yet no virucidal substances were demonstrated in the serum of recovered animals. Serum from hyperimmunized fowls administered intravenously in several doses of 5 cc. each had no therapeutic effect upon the established infection. Nor did injections of immune serum protect against a subsequent infection. Although no virucidal effect of immune serum was observed *in vitro*, provided the mixture was tested on scarified skin, Manteufel found, however, that an injection of a mixture of virus and immune serum does not cause an infection nor confer immunity. He infers from this that the virus in the presence of immune serum may be destroyed within the body of the normal fowl. These findings are confusing and the work should be repeated in the hope of throwing light on the subject.

In this connection are the important observations that a fowl recovered from the disease and refractory to reinoculation may still harbor the virus in the blood and internal organs for long periods. Sanfelice found the liver of pigeons infectious as long as 71 days after inoculation. The immunity may, therefore, be a non-sterile one. Without evidences of progressiveness in the disease, it is hardly analogous, as suggested by Lusena, to the non-sterile immunity encountered in tuberculosis and

syphilis. Immune animals, however, by harboring the virus are potential sources of infection.

The Relation of Epithelioma Contagiosum to Mammalian Pox

The suspicion of earlier writers that fowl-pox was closely related to or identical with variola and vaccinia has found recent expression in experimental work. Gins seems to have demonstrated that not only smallpox, but also sheep-pox and spontaneous goat- and swine-pox are convertible into vaccina by passage through rabbits.

van Heelsbergen by means of rabbit passage claims similarly to have converted fowl-pox virus into that of vaccinia. Also he seems to have succeeded in transmitting fowl-pox directly to the calf. Hens inoculated with vaccine virus developed a local immunity to fowl-pox. Toyoda confirmed the results both of Gins and of van Heelsbergen. He found, however, that hens inoculated with vaccine virus do not become completely protected against fowl-pox. He concludes that sheep-, fowl- and goat-pox have a common origin in variola.

The conclusions of van Heelsbergen and of Toyoda have not, however, been supported by the results of subsequent investigations. v. Toth and Ledingham successfully inoculated chickens with vaccine virus but found no evidence of immunity in recovered fowls to infection with the virus of fowl-pox, and *vice versa*. Loewenthal, Kadowaki and Kondo in a thorough investigation of the subject could find no cross-immunity induced by these viruses. They noted essential differences in the histological picture of the lesions of the two diseases in the fowl but did not discover Guarnieri bodies in those of vaccinia. Virucidal antibodies induced in the hen and rabbit with vaccine virus were active against this virus, but not against that of fowl-pox. No antibodies were found in the hen as a result of fowl-pox, either against this or the vaccine virus.

Andervont in a more recent exhaustive study obtained essentially the same results as those of Loewenthal and his coworkers. In addition he was able to demonstrate typical Guarnieri bodies in the epithelial cells of vaccinal lesions on the chicken's comb and cornea. He thus completely distinguished histologically as well as immunologically the effect of the two viruses in the same animal. Andervont's evidence, therefore, is convincingly opposed to the view that the viruses of epithelioma contagiosum and of vaccinia are identical or even closely related.

Relation of Epithelioma Contagiosum to Molluscum Contagiosum

It is hardly surprising that the similarity in the histological picture of molluscum contagiosum, as described by Virchow, to that of epithelioma contagiosum should have led Bollinger to the view that the two diseases have a common etiology. There is a striking similarity in the specific cellular inclusions of each disease, and Lipschütz has demonstrated in smears and in the cells of molluscum contagiosum minute bodies quite analogous to those previously discovered by Borrel in material from the lesions of fowl-pox. Csokor in 1884 accepted the view of the identity of the two diseases and claimed to have inoculated successfully on one occasion a cock's comb with the contents of a molluscum nodule. Within 10 days there appeared on the comb at the site of inoculation a bean-sized, mulberry-like nodule which microscopically proved to be identical with those of spontaneous epithelioma contagiosum. He transmitted the virus from this lesion successfully to other fowls. Sanfelice, however, was unable to infect pigeons with molluscum virus, and Pfeiffer and Juliusberg did not succeed in inoculating fowl-pox upon man. It has been shown by Marx and Sticker and by Juliusberg that the virus of each disease is filterable, but only that of molluscum contagiosum will pass through a porcelain (Chamberland F) filter. Lipschütz completely distinguishes the two diseases etiologically, clinically and pathologically. In view, however, of the similarity in the type of the cutaneous lesions, their closely analogous cytologic changes, and the apparently casual nature of attempted experimental work, it seems that the subject invites further investigation. The statement which occurs in certain current textbooks, that the two diseases are identical, is at the present time entirely unwarranted.

THEORETICAL CONSIDERATIONS

The nomenclature of this specific eruptive disease of fowls is very unsatisfactory. The name epithelioma contagiosum is obviously mistaken. Bollinger in proposing it did so in the belief that the nodules were true tumors. Although several of the filterable viruses stimulate a cellular hyperplasia, and some of them cause cellular growths which closely resemble neoplasms, a self-limited disease such as fowl-pox obviously at the present time has no place among tumors. The lesions are primarily an irritative hyperplasia but they rapidly undergo a recessive evolution ending in healing and immunity.

The appellation fowl-pox may be objected to on the ground that it connotes a more intimate relationship to variola and vaccinia than evidence justifies; nevertheless, in general, fowl-pox is much more congenial to the group of mammalian pox than to the tumors. If the term *pox* be used to mean an eruptive disease, fowl-pox has been applied intimately enough and long enough to this disease to be sufficiently definitive. The term chicken-pox is inadequate in the first place because it is not sufficiently inclusive, and secondly it is confusing with the common name of varicella. Likewise molluscum contagiosum of birds is confusing with human molluscum contagiosum, unless it can be shown that the two diseases are caused by strains of the same virus. The term fowl-pox altogether appears to be the most appropriate for general use.

At the present time facts regarding morphology are the vanguard of research concerning the etiology of fowl-pox. After many interpretational vicissitudes the specific epithelial inclusions have been shown to be composed of numbers of minute, uniform structures, having the appearance of microörganisms, embedded in a hyaline, often lipoid-containing medium which holds them together within the cell as a distinctive mass. It is not yet determined whether this medium is a product of the cell or of the microörganism it envelops, should the minute structures be accepted to be living things. Borrel recognized the presence of this material in stained preparations, clinging to each element like a mucinous capsule. A similar substance has been shown to encapsulate analogous bodies (those of molluscum contagiosum, rabies and others) and on this structural peculiarity v. Prowazek has based his classification of certain viruses as *Chlamydozoa*, while Lipschütz, in order to describe the minute coccus-like bodies of some of these diseases has used the generic term *Strongyloplasma*. He has named the virus of fowl-pox *Strongyloplasma avium*.

Cultural investigations have not yet confirmed the morphologic view of the microbic nature of the minute bodies, though this final demonstration is awaited with expectancy not only for the elucidation of the cause of fowl-pox but as a beacon to illuminate an entire group of diseases.

In the meantime additional facts may be gathered from immunological research which may serve as evidence in this connection. It should be determined whether the minute bodies are agglutinable and whether the capsular material is precipitable with specific immune sera. The rapid response of the skin of immune fowls to an inoculation of the virus deserves thorough study to determine whether it is of an allergic

nature or otherwise. The disease offers also an excellent opportunity for an investigation of the relation of immunity to the persistence of a virus within the refractory body, for the active agent of fowl-pox may reside in the immune animal for long periods.

It should be determined if possible whether the generalization of the virus is due to its active growth within the body fluids and internal tissues or to a passive dissemination from epithelial foci, within the ectodermal cells of which the virus is able to reproduce itself. The possibility of a specific and exclusively intracellular parasitism is a question of great importance in the pathology not only of fowl-pox but of all virus diseases of like nature. The apparent specificity of many viruses in their effect upon certain cells and tissues is one of the chief characteristics of these active agents. Of fundamental interest in this entire group of diseases is the relation of the respective viruses to the growth of cells and conversely to inhibition of cellular reproduction. For the study and solution of these and many other far-reaching problems, fowl-pox is most accessible and alluring.

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EXPLANATION OF PLATES

EXPLANATION OF PLATES

Plate 1

Fig. 1. An eruption on the comb 5 days after an intravenous injection of fowl-pox virus in an emulsion of a cutaneous tissue.

Fig. 2. A similar eruption 7 days after an intravenous injection.

Fig. 3. A secondary eruption on the head 12 days following an inoculation on the scarified skin of the breast. The cutaneous lesion was considerably traumatized by scraping.

Fig. 4. An early reaction on the comb 36 hours after inoculation. This lesion healed in 5 days. It represents the reaction of a partially or completely immune fowl.

Fig. 5. Lesions on the breast 6 days after inoculation. The feathers were plucked and the skin lightly scarified.

Fig. 6. A similar lesion of the breast which has become desiccated forming a hard black scab. This scab is rich in virus and later falls off.

Plate 2

Fig. 1. A section through a cutaneous lesion following an inoculation upon the scarified breast. Camera lucida drawing. The tissue was fixed in Regaud's fluid and stained by the acid fuchsin methyl green method. The inclusions and mitochondria are well seen. A reversal in position of the inclusions from the proximal to the distal portion of the cell is seen.

Fig. 2. Photomicrograph, magnification 1200 diameters. The inclusions first appear as condensations about vacuoles. The large mitochondria are evident.

Fig. 3. Magnification 1200 diameters. The large dark masses are virus bodies. The small round masses in the lower cell are nuclear extrusions, the so-called Benda bodies. Mitochondria are evident.

Plate 3

Fig. 1. Fresh preparation of material from a lesion suspended in physiological saline. Magnification 1200 diameters. The hyaline masses are the virus bodies freed from the cells.

Fig. 2. Fresh preparation in distilled water 1 hour. Magnification 1500 diameters. The virus bodies are greatly swollen, and numerous globules appear. Within these globules minute bodies in rapid Brownian motion may be seen in the fresh state. Some of these bodies adhere to the surface of the globule and appear in the photograph.

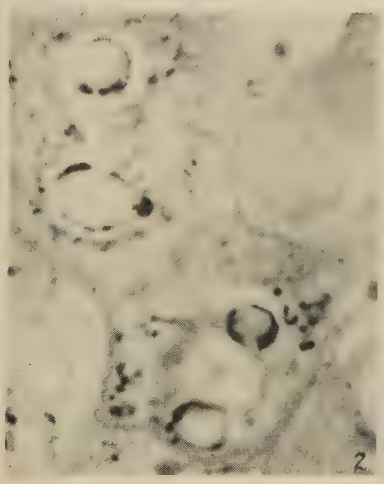
Fig. 3. A virus body broken up in a smear preparation. It is stained by the carbol-anilin-fuchsin method after mordanting with potassium permanganate. The two darkly staining bodies are bacteria. A faintly stained film represents the material in which the minute bodies are embedded to form the virus body. Magnification 2000 diameters.

Fig. 4. A drawing of Fig. 3, to show in greater contrast the encapsulating material of the virus body.

PLATE 1

PLATE 2

1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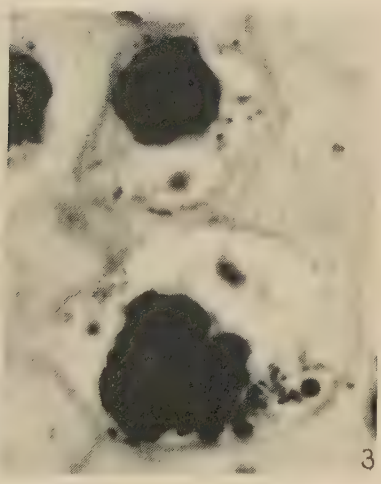
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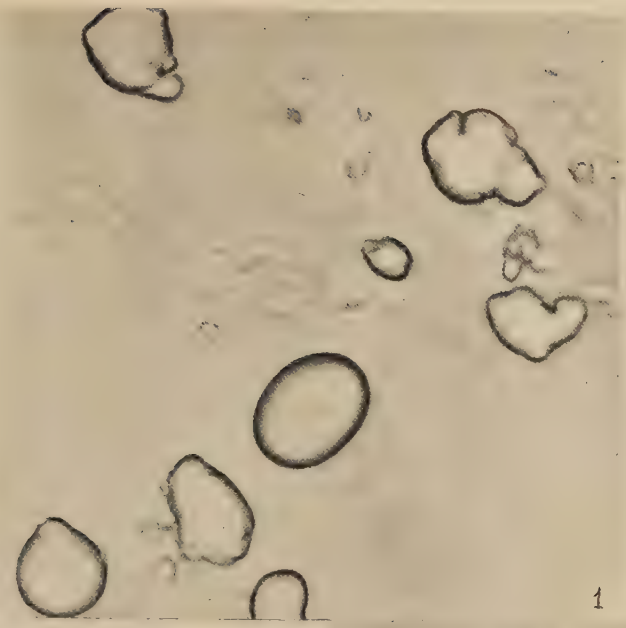
PLATE 3



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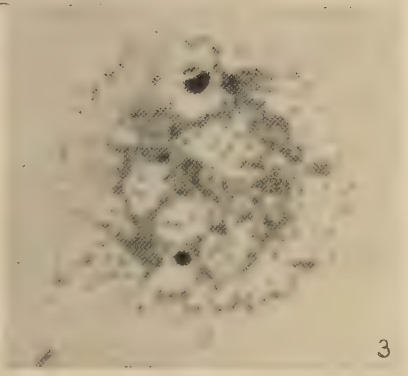

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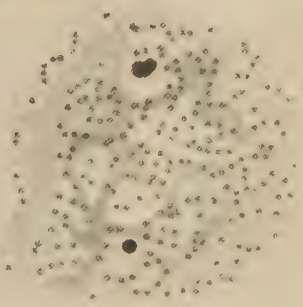

4

VIRUS DISEASES OF INSECTS

Sacbrood of Honey Bees

The Polyhedral Diseases

RUDOLF W. GLASER

CHAPTER VIII

VIRUS DISEASES OF INSECTS

GENERAL INTRODUCTION

Part I. Sacbrood of Honey Bees

Three general types of insect virus diseases have been described: (1) sacbrood of honey bees, (2) the polyhedral affections of certain lepidoptera, and (3) two diseases of *Pieris brassicae*, a cabbage butterfly. Insofar as is known, only the larval and pupal forms of the insects are susceptible. Moreover, each type of virus is specific for its own group; indeed, host specificity occurs within a single group. However, a number of distinguishing general characters exist which justify the arbitrary classification of three groups. Inclusion bodies undoubtedly play an important rôle in this classification. A histopathological study of bee larvae affected by sacbrood does not reveal the presence of inclusion bodies. In the polyhedral diseases of lepidopterous larvae, nuclear inclusions are found which are characteristic for this type of disease. In the third group or *Pieris* diseases, both nuclear and cytoplasmic changes occur, but are quite distinct from those included under group two.

The virus diseases of insects were encountered by human beings perhaps as early as 1857, but for at least a half century they were confused with bacterial, protozoan, and other affections. In fact all virus diseases suffered a similar fate up to the work of Iwanowski in 1892 on tobacco mosaic. v. Prowazek, in 1912, was the first to publish results of filtration experiments with an insect virus and concluded that in some cases healthy silkworms could be infected with bacteria-free Berkefeld filtrates of "grasserie" material. In 1913, White discovered a filterable virus disease of honey bees, and the same year Glaser and Chapman described one in gipsy moths. During 1915 and 1916, the latter authors tested a number of lepidopterous virus diseases. Komárek and Breindl in 1924 were the first to express a definite opinion in favor of the filterability of the virus of "Wipfelkrankheit," a disease of the nun moth. Paillot, in 1924 and 1926, described two new virus diseases of the cabbage butterfly.

A careful study of the virus diseases of insects is important for scientific and economic reasons. Insect virus material is easily procured and healthy insects can be readily obtained and cheaply reared in enormous numbers. Frequently in experimental work an increase in the number of animals intelligently used enhances the possibilities of important results. Therefore, insects offer remarkable opportunities for the quantitative study of viruses and should contribute much of fundamental importance to the general knowledge of virus diseases.

From the economic standpoint the subject also deserves serious attention. One need merely refer to the difficulties encountered by beekeepers when confronted by sacbrood or to those of the silk industry which in certain regions suffers annually from the ravages of grasserie. In these cases remedies and prophylactic measures are required. On the other hand, in combating some of our worst agricultural pests, such as the gipsy moth, nun moth, army worm, cutworm, tent caterpillar, and others, the best means for spreading diseases among them must be determined. Economic entomology has scored a number of notable successes in combating certain noxious insects with other insects and with fungus diseases that prey upon them. Bacterial diseases have also been used successfully a number of times, but this method of insect control is still unpopular because of the extraspecialized training required of the worker in this field. Nevertheless, bacterial diseases of insects, as well as virus affections, will undoubtedly constitute an important weapon for future economic entomology.

Sacbrood of Honey Bees

A disease of bees distinct from European and American foulbrood or other conditions was recognized by beekeepers probably as early as 1857. White (1) (1913) was the first investigator to make a careful study of the cause and nature of this malady which he named "sacbrood" to suggest the sac-like appearance of the dead larvae at the time they are most frequently seen by the beekeeper. In 1917 White (2) published a more detailed account of his extensive studies on this interesting disease. The following pages constitute an attempt to outline the essentials of those investigations.

Sacbrood is widely distributed geographically wherever bees are raised. The disease has been reported from nearly all sections of the United States and Canada as well as from Germany, Denmark, Switzerland, England, and Australia.

Sacbrood never attacks adult insects, but is an infectious and rather

benign disease of the brood of bees. It is insidious in its nature and somewhat transient in its character. The number of colonies that die as a direct result of sacbrood is comparatively small; the loss of individual bees, however, in the aggregate is enormous. This loss, naturally, tends to weaken a colony and makes the disease one of great economic importance.

The Appearance of Healthy Insects at the Age When They Die of Sacbrood

In a healthy comb in which brood is being reared, the larvae are arranged in such a way that capped and uncapped areas occur alternately and in more or less semicircular fashion. The larvae that die of sacbrood, with but few exceptions, do so within capped cells and at some time during the 4 days preceding metamorphosis. During the first 2 days of this prepupal period the larvae move about more or less within the cells and spin their cocoons. They are then comparatively quiet for about 2 days, lying on their dorsal sides extended lengthwise within their cells (Figs. 1, Plates 2 and 3). At the close of this 2-day period of rest, as a result of metamorphosis, the larvae change very rapidly to true pupae, assuming the outward form of adult bees.

Symptoms of Sacbrood

Although many larvae die of sacbrood during the first 2 days, or active period, of the 4-day prepupal period, by far the greater number of deaths occur during the last 2 days, the period of rest.

The presence of dead brood is usually the first symptom observed. An irregularity in the appearance of the brood nest (Plate 1) frequently attracts attention early in the examination. The strength of a colony in which the disease is present is often not noticeably diminished. Should many individuals of a brood become affected, however, the colony is weakened and this loss in strength soon becomes appreciable. Brood that dies of the disease does so almost invariably in capped cells. Dead larvae may be found, however, in uncapped cells, inasmuch as adult bees often remove the caps from cells containing dead individuals. It is rare to find a pupa dead of sacbrood. The dead larvae are found lying extended lengthwise with the dorsal side on the floor of the cell. Sacbrood, upon superficial examination, may be mistaken for some other abnormal condition of the brood. Therefore, a careful study of the postmortem appearance of larvae will make it possible to avoid any such confusion.

Appearance of Larvae Dead of Sacbrood

No signs in a larva dying of sacbrood have yet been discovered by which the exact time of death may be determined. As the larvae in this disease usually die during the time when they are motionless, lack of movement cannot be used as a sign of death. It is assumed that the larva is dead if it shows a change in color from bluish white to yellowish or if it exhibits indications of a change from the normal condition of turgidity to that of flaccidity. The appearance of a larva dead of sacbrood changes gradually from that of a living healthy larva to that of a dried residue—the "scale." For convenience of description, these changes in appearance are considered in five more or less arbitrary stages.

First stage.—(Figs. 2, Plates 2 and 3.) The dead larva has assumed a slightly yellowish appearance. This shade deepens somewhat, but does not become a deep yellow at this time. The lateral and posterior margins are still deeply notched and frequently appear quite transparent. This transparency is due to a watery-looking fluid beneath the cuticular portion of the body wall. The cuticle, which chiefly constitutes the sac that characterizes the disease sacbrood, is less easily ruptured at this time than in the healthy larva. When the body wall is broken the tissues of the larva, which constitute the contents of the sac, flow out. This fluid tissue mass is less milky in appearance than that from a normal larva. The granular character of the contents of the sac, which is marked in later stages of disintegration, is already in evidence. Upon microscopic examination the granular appearance is found to be due chiefly to fat cells.

Condition of the Virus in the First Stage.—When larvae of this stage are crushed, suspended in sirup and fed[1] to healthy bees, a large amount of sacbrood is readily produced, showing that the larval remains in this stage are particularly infectious. This is an important fact, as it is during this stage of disintegration that the larva is frequently removed piecemeal from the cell by the workers.

Second Stage.—(Figs. 3, Plates 2 and 3.) The color of the disintegrating larva has changed from the yellowish hue of the first stage to a brownish tint. The yellow, however, has not yet in all cases entirely vanished. The cuticular sac is now more readily observed and less easily broken. The contents consist of a granular brownish mass suspended in a watery fluid. Microscopic examination shows that the granular appearance is due in the main to the presence of disintegrating fat cells, which are changing slowly as the disorganization of the larva proceeds.

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[1] Having drawn some of the suspension into a capillary pipette, carefully touch the food in the cell surrounding the larva with the point of the pipette. A small amount of the suspension will flow out and mix with the food. Larvae approximately 2 days of age should be selected for feeding. A dozen or more should be fed in making an inoculation. The area of brood inoculated may be designated by marking on the brood frame, or by removing the brood from around the area inoculated.

Condition of the Virus.—The results of inoculations show that the remains of larvae at this stage of disintegration are still in some instances infectious.

Third Stage.—(Figs. 4, Plates 2 and 3.) The color of the dead larva of this stage is brown, that of the anterior third being a deeper shade than that of the other two-thirds. A wrinkling of the surface appears and is an indication that the remains are drying. That the watery content of the sac is being lessened through evaporation is also evidenced by the diminution of the quantity of the watery-appearing substance seen at the lateral margins of the middle and posterior thirds. The lateral view of the larva in the third stage shows that it still maintains, in a general way, the form and markings of the normal larva. The turgidity is gone, although the position in the cell is very much as it is in healthy larvae.

Consistency of the Sac and Its Contents.—The appearance of the remains of the larva in the third stage of disintegration best characterizes the disease sacbrood. The cuticular sac is now quite tough, permitting the removal of the larva from the cell with considerable ease and with little danger of its being torn. The content of the sac is a granular mass, brownish in color, and is suspended in a comparatively small quantity of clear watery fluid. Upon microscopic examination the mass is still found to consist chiefly of disintegrating fat cells.

Condition of the Virus.—When the larval remains in this stage of disintegration are crushed and fed in sirup to healthy colonies no sacbrood is produced, indicating that the dead larvae at this stage are not infectious.

Fourth Stage.—(Figs. 5, Plates 2 and 3.) The brown color of the larval remains has further deepened, the anterior third being ordinarily much darker than the other two-thirds. The marked evidence of drying now present characterizes this stage. The subcuticular fluid is no longer evident.

Consistency of the Contents of the Sac.—Upon tearing the sac, the contents are found to be less fluid than in preceding stages. The disintegrating tissue mass is still granular in appearance and as the drying proceeds further the contents of the sac become paste-like in consistency.

Condition of the Virus in the Fourth Stage.—As in the preceding stage, the larval remains do not seem to be infectious.

Fifth Stage.—(Figs. 6, Plates 2 and 3.) The dead larva at this time has lost all its moisture by evaporation, leaving a dry mummy-like mass known as the "scale," which can be removed intact from the cell. Indeed, when very dry, many of them can be shaken from the brood comb. When out of the cell, they vary markedly in appearance. In general, the anterior third is usually of a deeper brown than the other two-thirds. The dorsal side of the middle and of the posterior thirds is shaped to conform to the floor of the cell, being in general convex, with a surface that is smooth and polished. The margin is thin and wavy. The anterior third and the lateral sides of the middle and posterior thirds are turned upward, the ventral surface is concave, and the posterior side convex. The scale in general resembles a boat and may be styled "gondola-shaped."

Condition of the Virus in the Scale.—The scales in sacbrood, when fed to healthy bees, have shown no evidence of being infectious. The length of time that dead larvae are permitted to remain in the cells by the bees before they are removed varies. They may be removed soon after death, they may remain until or after they have become a dry scale, or they may be removed at any intervening stage in their disintegration. Not infrequently they are permitted to remain to or

through the stage described above as the third stage. That the dead larvae are permitted to remain within the cells frequently for weeks is in part the cause of the irregularity observed in the appearance of the brood combs (Plate 1).

The Histopathology of Larvae Dead of Sacbrood

A section through a larva dead of sacbrood shows that the fat tissue constitutes the greater portion of the body. The fat cells are comparatively large, irregular in outline, with an irregularly shaped nucleus and enclose black-staining spherical bodies of various sizes[2] (Text-fig. 1). The presence of these cells chiefly causes the granular appearance of the contents of the dead larvae.

The molt skin is at a considerable distance from the hypodermis. Another cuticula is already quite well formed and lies near the hypodermis. Between the two cuticulae, during the earlier stages of disintegration a

TEXT-FIG. 1. Fat cell with irregular outline, irregular nucleus, and deeply staining bodies in cytoplasm. (After White.)

considerable space intervenes which is filled with a watery fluid. That the fluid is not water is shown by the presence of coagulated material in the sections.

The body wall of the larva is composed of the cuticula, the hypodermis, and the basement membrane. The comparatively small hypodermal cells may be present in the contents of the larval remains. Similar cells are found in the tracheal walls, but constitute only a small portion of the contents of the sac. There are many other cellular elements found in the disintegrating mass, some of which contribute to the granular appearance. Among these are the oenocytes, cells larger than the fat cells, but comparatively few in number.

[2] These probably represent the so-called protein bodies—metabolic products often found within the cytoplasm of the fat cells of insects prior to and during metamorphosis.

The molt skin seen is probably the one shed normally about 3 days after the larva is capped. The cuticula, already quite well formed, is probably the one which normally would have entered into the formation of the molt skin cast at the time the larva or semipupa changes to a pupa. This molt skin primarily constitutes the sac which encloses the disintegrating larval mass in sacbrood. The cuticula probably assists somewhat at times. The presence of the subcuticular fluid becomes more intelligible in the light of these facts. Larvae dying of sacbrood at an earlier or later period in their development will present an appearance varying somewhat from that first described.

Contrasted with that of a feeding larva, the midgut of a larva at the age when it dies of sacbrood is small. The cells lining the wall of the organ vary considerably in size and shape, depending upon the exact time when death occurs. In contrast to the low cells of the stomach wall in younger larvae, the cells at this later period are much elongated. These cells are also at times found in the disintegrating granular mass present in the larval remains.

The various organs of the body contribute to the disintegrating larval mass. At the period when a larva dies of the disease, the cellular changes accompanying metamorphosis are particularly marked. This transformation with its disintegrative changes also introduces various cellular elements into the disorganized larval remains.

The granular mass observed is, therefore, a composite affair. Upon examining it microscopically, one finds that the granular appearance is primarily due to fat cells suspended in a liquid which seems to be chiefly blood, or at least, derived from the blood, although most probably augmented by other body fluids and possibly by a liquefaction of some of the tissues. The granular mass suspended in a watery fluid, as a symptom of sacbrood, is thus rendered more comprehensible.

Cytoplasmic or nuclear structures, commonly termed cellular inclusions or reaction bodies, present in many virus diseases of other animals, have not been found in sacbrood larvae.

Cause of Sacbrood

Predisposing Causes.—Sacbrood seems to be influenced by a number of predisposing causes, and age appears to be the most striking of the apparent possibilities. Adult bees are not susceptible to the disease and pupae are rarely affected. If a pupa succumbs it is quite soon after its transformation from the larval stage. Sacbrood is primarily a larval disease and when a larva dies it does so invariably after capping and

usually during the 2-day period immediately preceding the time for it to change to a pupa. In regard to sex as a predisposing influence, worker and drone larvae are easily infected. Queen larvae are apparently also susceptible, but this fact has not yet been definitely established. That different races of bees vary in their susceptibility to sacbrood seems likely, but proof for this contention is still lacking. Sacbrood is so widespread geographically that it does not seem likely that climate plays an important rôle. While the disease is more prevalent and occurs with greater severity in the spring, it is thought that various factors produce this result and that it is not influenced directly by the season of the year. Lastly, food has nothing to do with predisposing bees to the infection.

Exciting Cause.—That sacbrood is an infectious disease was demonstrated by White in 1913. This was accomplished by feeding to healthy colonies the crushed tissues of larvae dead of sacbrood, suspended in sirup. The experiments were performed under various conditions and it was found that the disease can be produced at will. Thus the infectious nature of the disease was proved.

In the crushed larval mass no microörganisms were found either microscopically or culturally to which the infection could be attributed. Some experiments with Berkefeld filtrates were then instituted. Larvae which had been dead of sacbrood only a few days were picked from the brood comb and crushed. The crushed mass was added to water in the proportion of 1 part larval mass to 10 parts water. This aqueous suspension was allowed to stand for some hours, preferably overnight. To remove the large fragments of larval tissues the suspension was filtered through paper. The material thus obtained was then filtered by gravity through a Berkefeld candle.

To determine whether any visible microörganisms were present in this last filtrate, it was examined microscopically and culturally. When it was found to be apparently free from such microörganisms a quantity of it was added to sirup and the mixture fed to healthy colonies. When all was properly done, sacbrood appeared in the inoculated colonies. White also used Pasteur-Chamberland filtrates obtained by the aid of pressure. Such filtrates when fed to healthy colonies also produced the disease.

The Weakening Effect of Sacbrood upon a Colony

The results of inoculation experiments demonstrated that sacbrood may weaken a colony. A colony may be destroyed by the disease, or it

may recover, gain in strength, and pass through the winter successfully. Each year from 1912 to 1917 two or more colonies were fed sacbrood virus at intervals during the brood-rearing season in order to obtain material for experimental purposes. The inoculated colonies in all instances showed a tendency to become weakened as a result of such treatment. The death of the worker larvae is the primary cause for the weakness resulting from the disease. Since dead sacbrood larvae remain in the cells for weeks, as they not infrequently do, the strength of the colony is also lessened by a reduction in the capacity of the brood nest for brood rearing.

Amount of Virus Required to Produce the Disease

Until the virus has been cultivated on artificial media, it will be impossible to definitely determine the minimum quantity necessary to produce a moderate infection. It has been found, however, that the virus in a single larva recently dead of the disease was sufficient to produce infection and death of at least 3000 healthy larvae in less than 1 week.

The Effects of Physical and Chemical Agents on the Virus

Although the virus may increase with great rapidity, fortunately it is quite as readily destroyed. Nature supplies many means by which this may be accomplished. While theoretically a sufficient amount of virus may be produced within 1 month to inoculate all the bees in existence, within another month, if exposed to many forces of nature, practically all such virus would be destroyed. This fact constitutes one of the chief reasons for the comparatively rapid recovery of colonies from the disease.

Sacbrood virus suspended in water is destroyed in 10 minutes at a temperature of 59°C. When suspended in glycerol or in honey it is destroyed in 10 minutes at 70–73°C. The virus withstands drying at room temperature for approximately 3 weeks when it is shielded from light. Negative inoculation results obtained from the use of larval material which was dried for more than 7 months tends to eliminate the possibility that the virus possesses a resting stage. Similar experiments were performed to see how long the virus would remain infectious at outdoor temperature and at incubator temperature (37°C.). The results were approximately the same as those obtained when it was dried at room temperature. The time was somewhat less, however, in the case of drying at incubator temperature. Preliminary tests also in-

dicated that when the virus was mixed with pollen and permitted to dry, the period during which it remained infectious was increased only slightly.

When the virus is first dried at room temperature for a few hours and then exposed to the direct rays of the sun, it is inactivated in from 4 to 7 hours. The experiments indicate that much of the virus is rendered innocuous during a 2-hour exposure. When the virus suspended in water is exposed to sun rays, it is destroyed in from 4 to 6 hours; when suspended in honey, in from 4 to 6 hours. It might be well to state that these suspensions did not reach by several degrees the temperature (59°C.) at which the virus is readily inactivated. The time given represents that required for the inactivation of all of the virus used in each experiment.

In devising methods for the treatment of sacbrood it is of special interest to know how long the virus will remain virulent when it is suspended in honey. Under such conditions, when kept at room temperature, the virus remained active for 3 weeks, but was rendered entirely innocuous before the end of the fifth week.

In the presence of the fermentation, at room temperature, of a 10 per cent cane-sugar solution, the virus is inactivated in from 3 to 5 days. In the presence of the fermentation of a 20 per cent honey solution at outdoor temperature, it ceases to be infectious in 6 days. By outdoor temperature warm weather is implied. During cooler weather the period of time is naturally somewhat increased. In the presence of any putrefactive process the virus is inactive after 7 to 10 days.

The active agent shows a marked resistance to the disinfecting power of carbolic acid. Under experimental conditions the virus resisted its action for more than 3 weeks in 0.5, 1, and 2 per cent aqueous solutions.

Modes of Transmission of Sacbrood

The transmission of a brood disease must be considered to occur (1) from diseased to healthy larvae within a colony and (2) from a diseased colony to a healthy one.

It has been shown experimentally that the virus produces the disease when it is added directly to the food of young larvae or when it is mixed with sirup and fed to a colony. From this fact it is fair to assume that sacbrood may result whenever the food or water used by the bees contains active virus. Bees have a tendency to remove diseased or dead larvae from the cells. When the removal is attempted about the time of death it is done piecemeal. Due to this habit, the mouth parts of the

workers undoubtedly become contaminated. Within the hive, therefore, the disease may be transmitted to healthy larvae more or less directly in this way.

Should any material containing the virus reach the water supply of the bees, it is within the range of possibility that some of the active virus might be returned to the hive and reach healthy young larvae. Indeed, it seems that the contamination of the water supply presents a greater source of danger than do flowers. Bees drifting or straying from infected colonies to healthy ones must be considered as possible transmitters. The disease, however, is not spread to any great extent in this way. Sacbrood has a tendency to weaken a colony in which it is present. Frequently this weakness is noticeable and often marked. Robbing by stronger colonies, which often occurs at such a time, may result in the transmission of the virus directly to healthy colonies. The modes of transmission of sacbrood within the colony and from colony to colony are not as yet completely determined. In what manner the virus is carried over from one brood-rearing season to another is one of the many problems still awaiting solution.

Differential Diagnosis of Sacbrood from Other Brood Diseases

The diagnosis of sacbrood can be made from the symptoms already described. However, the disease must often be differentiated from other brood disorders.[3]

American foulbrood (4) may be recognized by the peculiar odor of the brood combs. The body wall of the larval and pupal remains is easily ruptured, and the decaying mass becomes viscid, giving the appearance popularly referred to as "ropiness." The scale adheres quite firmly to the floor of the cell. The presence of *B. larvae* in the brood dead of the disease is a positive means by which it may be differentiated from sacbrood.

European foulbrood (5) may be recognized by the fact that the lavae as a rule die while coiled in the cell and before an endwise position is assumed. In the majority of instances, therefore, death takes place before the cells are capped. The sac-like appearance and the granular consistency of the disintegrating mass characterizing larvae dead of sacbrood are absent. Microscopically, a large number of bacteria are found in larvae dead of European foulbrood. The presence of *B. pluton* is a positive means by which European foulbrood may be recognized.

[3] Nosema disease (3), a protozoan disease, which only attacks adult bees, need not be considered in a differential diagnosis of brood affections.

Sacbrood must also be differentiated from other conditions referred to as chilled brood, overheated brood, and starved brood, which are occasionally encountered. This can be accomplished by a comparison of the history of the cases and the symptoms presented by the different conditions with those of sacbrood. It should again be emphasized, however, that the sac-like remains characteristic of sacbrood are not present in other conditions.

Prognosis

Whether a larva once infected with sacbrood ever recovers is not known. The tendency in an affected colony is to recover from the disease. Colonies which during the spring months show the presence of the disease, by midsummer or earlier may, and very frequently do, contain no diseased brood. Experimentally it is possible to destroy a colony by feeding it repeatedly with the virus, and beekeepers report that the disease sometimes destroys colonies in their apiaries. The percentage of colonies, however, that actually "die out" as a direct result of the disease is small. The weakening of the colony in the spring of the year not only reduces or entirely eliminates the profits for the season, but may also injure it to such an extent that it does not pass through the winter well.

The Treatment of Sacbrood

That the virus of sacbrood remains virulent in larvae dead of the disease for less than 1 month; that it remains virulent in honey approximately 1 month; that when mixed with pollen it ceases to be virulent after about 1 month; and that in drying no virulence is to be expected after 1 month, are facts that account in a large measure for the strong tendency to recover from the disease manifested by the colony and that furnish information concerning the use of combs from sacbrood bees. From the results it may be concluded that it is better, theoretically, to store combs from sacbrood colonies for 1 or 2 months before they are again used, provided such storing entails no particular inconvenience or financial loss to the beekeeper. At any event, it is comforting to know that it is never necessary to destroy the combs from sacbrood colonies on account of the disease.

The experimental results on the destruction of the virus through heating, fermentation, putrefaction, drying, and direct sunlight should assist materially in the solution of the problem of the transmission of sacbrood, and should prove helpful in devising efficient methods for the control of the disease.

No fear need be entertained in practical apiculture that the disease will be transmitted by the hands or clothing of the operator, by the tools used about the apiary, through the medium of the wind, or by the queen. It would seem at all times superfluous to flame or burn the inside of the hive or to treat the ground about a hive containing an infected colony. Little danger exists that the virus will be transmitted to flowers visited by workers from sacbrood colonies and later to healthy colonies by other bees.

Theoretically, it is possible that the disease may be transmitted through a contamination of the water supply. Whether infection ever takes place in this manner, however, is not yet known. If the disease is ever transmitted in this way it probably occurs more frequently in the spring of the year than at any other season.

To summarize this section, it may be stated that very little is really known concerning the treatment and prophylaxis of sacbrood. Whether the application of serums, vaccines, or drugs is possible and practicable will remain problematical until further knowledge of these matters is obtained.

SUMMARY

1. Sacbrood is an infectious disease of the brood of bees.

2. Adult bees are not susceptible.

3. The infecting agent is a filterable virus which up to the present time has not been cultivated on artificial media.

4. A colony of bees may be inoculated by feeding it sirup or honey containing the virus.

5. The quantity of virus in a single larva recently dead of the disease is sufficient to produce quite a large amount of sacbrood in a colony.

6. The incubation period is approximately 6 days.

7. By inoculation the disease may be reproduced at any season of the year during which brood is being reared.

8. The spontaneous disease is more often encountered during the first half of the brood-rearing season than during the second half.

9. It occurs among bees in localities having as wide a range of climatic conditions, at least, as are found in the United States.

10. The course of the disease is not greatly affected by the character or quantity of the food obtained and used by the bees.

11. Larval remains recently dead of the disease prove to be very infectious. Dead larvae which have been in the brood comb more than 1 month are apparently non-infectious.

12. Colonies possess a strong tendency to recover spontaneously from the disease.

13. The effects of various physical and chemical agents on the virus are outlined.

14. The symptoms of sacbrood and the differential diagnosis of the disease from other brood diseases are described.

15. The histopathology of larvae dead from sacbrood is described. No cellular inclusions or reaction bodies are found within any of the tissues.

16. Varying factors may enter into many of the problems discussed and may alter the results obtained. Therefore, the results given at this time should be considered only approximately correct from a technical point of view. In the section dealing with the destruction of the virus, the results are sufficiently exact for application by the beekeeper, but the time element indicated by the experiments should be increased somewhat.

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EXPLANATION OF PLATES

EXPLANATION OF PLATES

Photograph and figures after G. F. White

Plate 1

Brood frame containing sacbrood, taken from an experimental colony in which the disease was produced by feeding the virus.

Plate 2

Fig. 1. End view of healthy worker larva in normal position in the cell. Cap torn and turned aside with forceps.

Fig. 2. First stage: Larva showing first symptoms of sacbrood and presenting the dorsal view of the anterior third. Cap removed artificially.

Fig. 3. Second stage: Dorsal view of anterior third of a larva dead of sacbrood.

Fig. 4. Third stage: Dorsal view of anterior third of larva dead of sacbrood.

Fig. 5. Fourth stage: Remains of larva dead of sacbrood.

Fig. 6. Fifth stage: Scale, or larval remains, in sacbrood as seen on looking into the cell.

Figures enlarged approximately 5½ diameters.

Plate 3

Fig. 1. Healthy larva and cell viewed from above and at an angle.

Fig. 2. First stage: Ventral view of larva dead of sacbrood as seen from above and at an angle, giving a ventral view of all three thirds. Cap torn across.

Fig. 3. Second stage: Larva dead of sacbrood, ventral view.

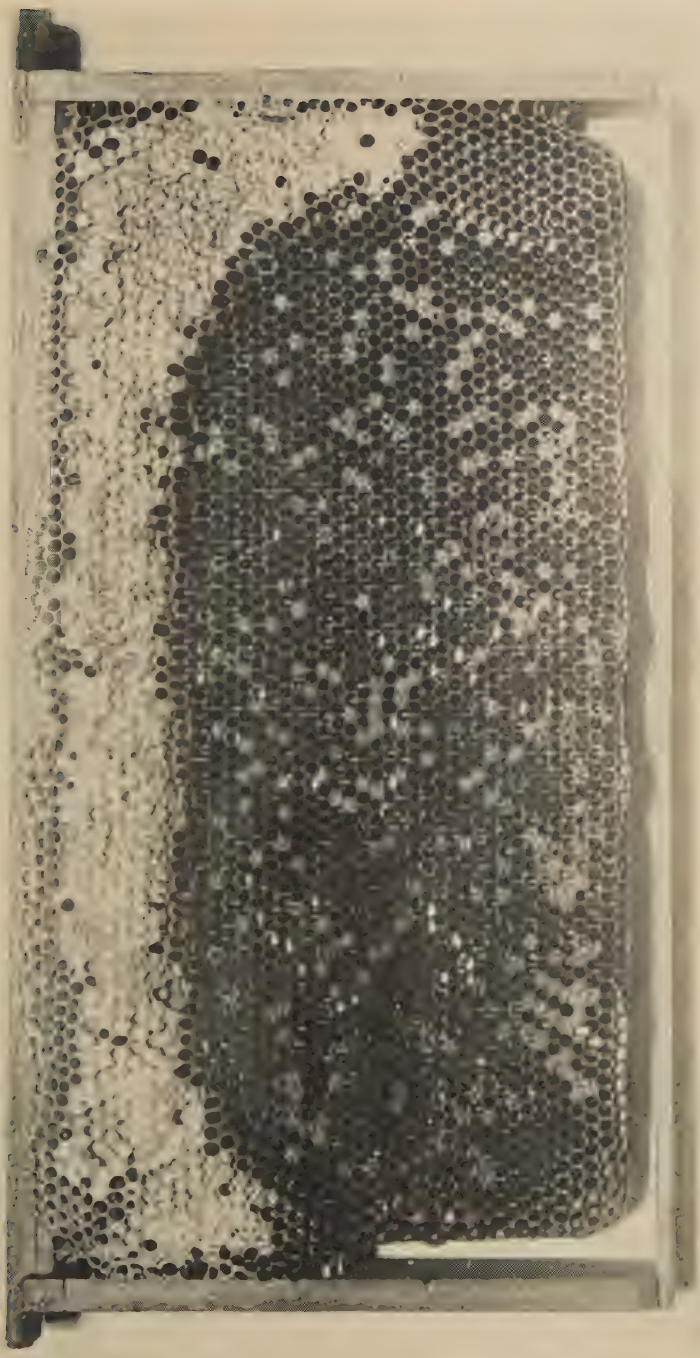
Fig. 4. Third stage: Larva dead of sacbrood, ventral view.

Fig. 5. Fourth stage: Remains of larva dead of sacbrood, ventral view.

Fig. 6. Fifth stage: Scale, or larval remains, in sacbrood viewed at an angle from above.

Figures enlarged approximately 4 diameters.

PLATE 1



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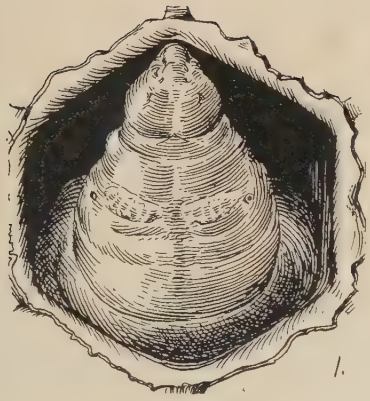
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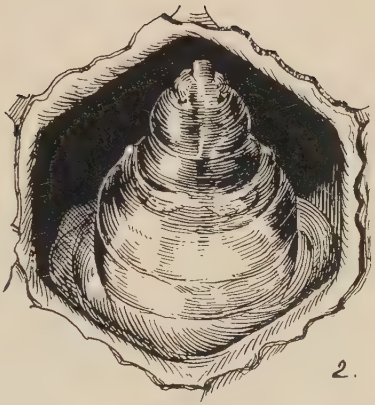
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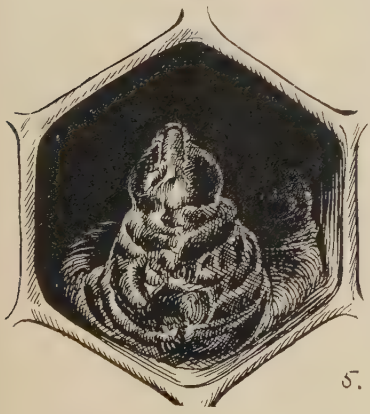
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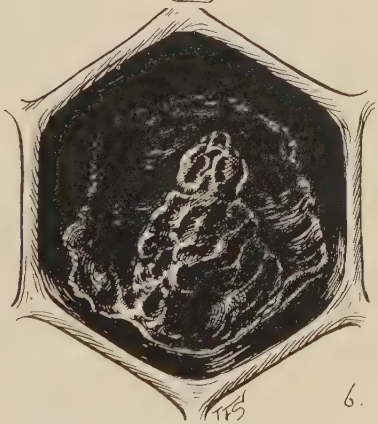
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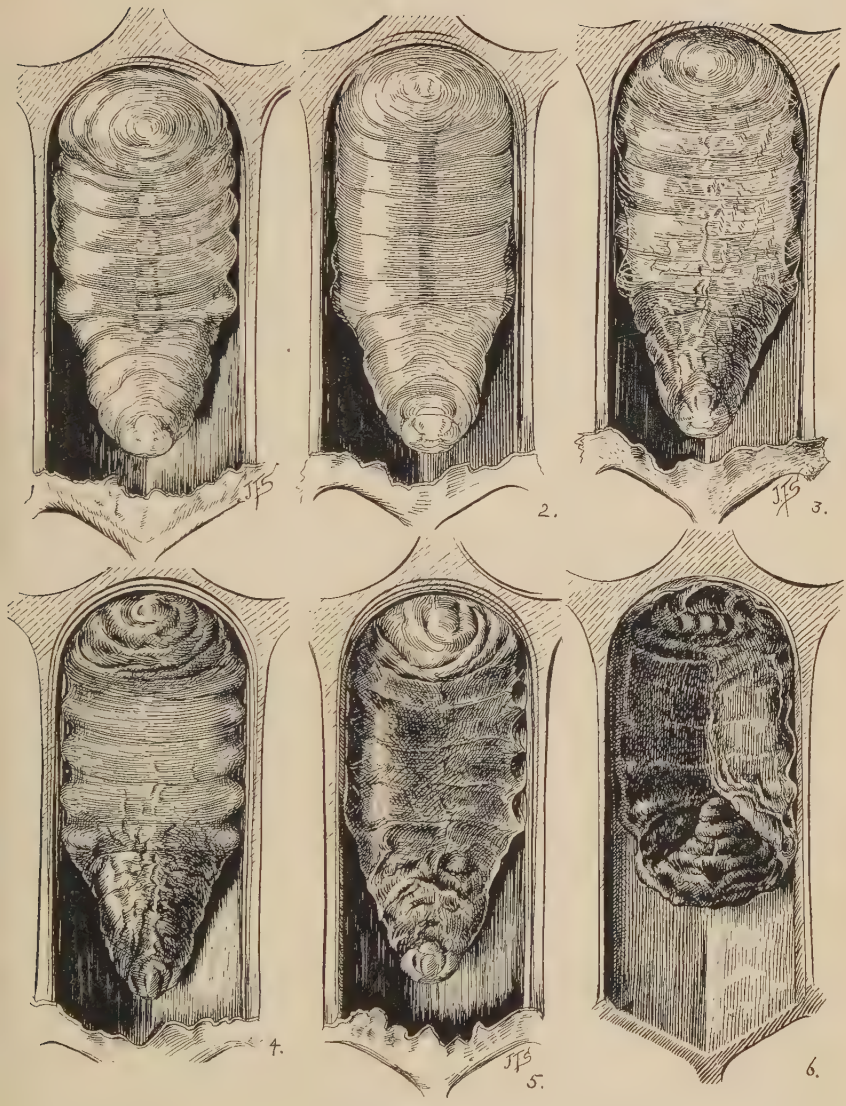
PLATE 3



CHAPTER VIII

VIRUS DISEASES OF INSECTS

Part II. The Polyhedral Diseases

The polyhedral affections of insects constitute a group of diseases that, insofar as is known, attack only the larval and pupal forms of certain lepidoptera. These maladies are widespread, especially among the moths of America, Europe, Asia, and probably elsewhere, and affect many of our worst pests, producing at times devastating epidemics among them. Such epidemics contribute more towards the control of certain noxious caterpillars than do the combined efforts of all their hymenopterous and dipterous parasites. Unfortunately the silkworm is also subject to one of these affections. The silk industry in certain regions suffers because of its depredations, inasmuch as no satisfactory remedy for its control has yet been devised.

In the United States and Canada the polyhedral maladies, no matter in which species they may occur, are commonly termed "wilt" or simply "polyhedral disease," to distinguish this type of affection from "flacherie" or other conditions. In Europe "Polyederkrankheit" or "Wipfelkrankheit" designates a similar disease in nun-moth caterpillars. When appearing in silkworms it is termed "grasserie," "jaundice," "Gelbsucht," or "Fettsucht."

Polyhedral diseases have been described in many different species of insects, often from widely divergent orders. This confusion arose because of faulty diagnosis based on an erroneous knowledge of the histopathology and etiology of these interesting maladies. On careful analysis, it is found that the polyhedral diseases are restricted to the lepidoptera and with two exceptions to the moth group. All species of moths, however, are not susceptible. The following list comprises those larvae that are naturally and experimentally susceptible to one of the polyhedral diseases.

Family of Moths	*Common Name*
Saturnidae	
Hemileuca maia Drur.	buck moth
Arctiidae	
Apantesis virgo L.	virgin tiger moth

Family of Moths	*Common Name*
Noctuidae	
Leucania unipuncta Haw.	army worm
Noctua clandestina Harris	cutworm
Autographa brassicae Riley	cabbage worm
Lymantridae	
Porthetria dispar L.	gipsy moth
Lymantria monacha L.	nun moth
Orgyia leucostigma S. and A.	white marked tussock moth
Lasiocampidae	
Malacosoma americana Fab.	tent caterpillar
Malacosoma disstria Hbn.	forest tent caterpillar
Bombycidae	
Bombyx mori L.	silkworm
Dioptidae	
Phryganidia californica Packard	shade tree pest
Family of Butterflies	
Papilionidae	
Colias philodice Godart	clover sulphur
Pontia rapae Sch.	cabbage worm

Diagnosis

The polyhedral viruses have been most carefully studied in three species, namely, in the silkworm (*Bombyx mori* L.), in the nun moth (*Lymantria monacha* L.), and in the gipsy moth (*Porthetria dispar* L.). The disease produced in silkworms is known as grasserie, in Italy, jaundice in France, and Gelbsucht or Fettsucht in Germany. Affected worms are usually covered with lemon-yellow blotches a few days before death, are inactive, and cease to eat. Before dying, the skin assumes an opaque, shiny, yellowish appearance (*glanzraupen*, *luisettes*). The Italians name such worms *vacca* (milk cows) and the Germans *Fettraupen*, since the animals are somewhat swollen when in this condition. Affected nun-moth larvae show few recognizable symptoms excepting loss of appetite and a tendency to migrate to the tree tops or Wipfel where, hanging by their prolegs, they die in large numbers. Due to this habit the disease has been known in the German-speaking countries as "Wipfelkrankheit." In America, the polyhedral disease of gipsy moths and tent caterpillars is termed "wilt." In these two species few recognizable symptoms exist excepting inactivity and loss of appetite. At the time of death and shortly after, both forms assume a shiny aspect, are entirely flaccid and without offensive odor (Plate 1).

The blood of healthy larvae has a clear yellow color. During the last

stages of disease it has a cloudy, yellowish white appearance in silkworms, and a cloudy, brownish color in the other forms. The skin of such larvae ruptures very easily on handling, permitting the blood to escape.

It is extremely difficult to remove the cadavers without rupturing their skin and liberating the liquid contents. This is due to the fact that within a few hours after death all of the tissues are disorganized. If a film from the fluid of dead caterpillars is examined microscopically, it will be found to contain the elements of disorganized tissues and myriads of polyhedral bodies of various sizes (Plate 2, Figs. 1 and 2; Plate 3, Fig. 1). In the silkworm the average polyhedron measures 3–5 μ in diameter, although individuals have been found as small as 0.5 μ and as large as 15 μ. The average polyhedron of the gipsy moth measures 3.4 μ in diameter; that of the nun moth 2.65 μ; that of the tent caterpillar 2.6 μ; and so on down the scale until one comes to those of *Phryganidia californica* and tussock-moth larvae which measure 1.6 μ and 1.5 μ in diameter respectively. The polyhedra occur singly or in pairs, are very refractive (not double refracting under the polariscope) and superficially resemble crystals. By manipulating the slide and coverslip, the bodies can be turned over, so that excellent views of all their faces can be obtained. Their shape varies as does their size, and in silkworms 5, 6, 7, 8, and more faces are common (Plate 3, Fig. 2). The corners are often sharp and angular. In gipsy larvae and tent caterpillars the form is that of a polyhedron with more or less rounded corners (Plate 3, Fig. 3). They never assume the shape of a perfect sphere. Upon careful examination the bodies seem to have a dense center differentiated from a somewhat lighter peripheral mass. Often, within the bodies, concentric layers like those of potato starch grains are observable, indicating that growth by accretion occurs. When pressure is applied, the polyhedra crack very readily into a number of pieces and often without application of pressure, fragmentation may be observed to occur somewhat more slowly. In the latter case a notch appears at one side of the polyhedron, which gradually lengthens into a line progressing slowly toward the other side, much like the cracking of ice. Usually, before the line has completely separated the two halves, other cracks appear, and soon the entire polyhedron is divided into a number of pieces, which may separate or stick together in a rosette-like fashion (Plate 3, Figs. 4–9). The bodies have been carefully observed during the process of fragmentation, but nothing has ever been seen to escape from them. Fat globules and urate crystals have been confused with

polyhedra by the inexperienced. This should not occur, however, inasmuch as the fat drops are perfectly spherical, stain with Sudan III, and are soluble in ether, while polyhedra do not stain with Sudan III and are insoluble in ether.

A stained film from a larva, dead of grasserie or wilt, reveals polyhedral bodies, fat drops, urates and other crystals, cellular débris, hairs, setae, food particles, and pigment granules. The pigment granules, plentiful in a pigmented form like the gipsy-moth larva, must not be confused with bacteria, to which they bear a superficial resemblance. As a matter of fact, larvae that have recently died are almost devoid of bacteria, and in many cases none can be found. If present, the larva has been dead for some time and bacteria have entered through various channels.

The polyhedra are very resistant towards stains in general, and usually take the color only around the periphery, unless the stain is applied for a long time following a mordant, or unless some drastic method is used, such as, steaming with carbol fuchsin. When they react, they stain uniformly, or they may show structures that simulate small refractive granules and striations. The dark field reveals these granulations very well. As previously mentioned, however, when the bodies are fragmented nothing that can be correlated with the etiological agent of the disease is seen to escape.

The polyhedra are heavier than water and consequently can be obtained in bulk by centrifuging aqueous emulsions of diseased material. By repeated washing and centrifuging the fat and cellular débris can be eliminated and the bodies thus may be obtained in a fairly clean condition. They do not dissolve in hot or cold water, carbon bisulphide, alcohol, ether, chloroform, glycerol, benzine, hydrogen peroxide, benzol, saponin, or sapotoxin, but are soluble when heated in strong solutions of acids and weak solutions of alkalis. They are soluble in pepsin hydrochloric acid mixtures, and in natural gastric juice. They do not blacken with osmic acid and do not color with Sudan III. Therefore they contain no fat. Picric acid stains them yellow, showing that they contain protein material. The polyhedra, in view of their reactions, are regarded as nucleoprotein crystal-like degeneration products of the disease. They give all the protein color reactions, are soluble in weak alkali, and are precipitated from this solution by acids. The nucleins which have a high phosphorus content are not decomposed by gastric juice and are obtained as an insoluble residue after the artificial digestion of the nucleoproteins with pepsin (ammonium-phosphomolybdate

test). Iron has also been quantitatively determined to be a constituent of the polyhedra. On dissolving polyhedra in weak alkali and after dialyzing away the alkali, one can obtain three fractional precipitations with magnesium sulphate or sodium chloride.

Historical Review

The early literature apparently confused the polyhedral affections with other insect diseases. Later, Wahl, followed by v. Prowazek and Escherich, however, clearly showed that these diseases are entirely distinct from such maladies as "muscardine," "pébrine," "flacherie," "lethargia," "maladie de morts blancs," Schlaffsucht," Faulsucht," etc. Since the polyhedral diseases have been most carefully studied in the nun- and gipsy-moth larvae and in the silkworm, a general account of these studies is absolutely necessary for a proper appreciation of the entire subject.

Wipfelkrankheit

Wipfelkrankheit of the larvae of *Lymantria monacha* was first carefully studied by O. Hofmann (16) and v. Tubeuf (31, 32) during a nun-moth epidemic during the years 1889 and 1892. Both Hofmann (1891) and v. Tubeuf (1892) considered Wipfelkrankheit to be bacterial in nature; to the former investigator a *Bacillus "B"* seemed important, while to the latter a bacterium abundant in the digestive tract and named by him *Bact. monachae* appeared to deserve particular attention. The polyhedral bodies were seen by both workers and classified as reaction products. Wachtl and Kornauth (34), in 1893, found that the polyhedral bodies in nun larvae originate within cells. These workers were the first to realize that the bodies have great diagnostic value, inasmuch as they are always present in enormous numbers in caterpillars afflicted with Wipfelkrankheit. The bacteriological findings of Wachtl and Kornauth had no etiological significance; the same is true of Tangl's work (30) in 1893.

Eckstein (8) in 1894 considered *Bacillus "B"* of Hofmann and *Bact. monachae* of v. Tubeuf identical. v. Tubeuf himself in 1911 (33), however, reversed his opinion on the specific cause of the disease, and concluded that Wipfelkrankheit develops when a variety of intestinal bacteria become dominant.

Wahl (35) in a series of articles from 1909 to 1911 critically reviewed the entire subject and brought a certain degree of order out of chaos. He demonstrated that the disease is an infection apparently not bacterial in nature, and assigned the cause to an unknown virus. Wahl showed that the polyhedra originate within the nuclei of certain cells, especially those of fat and blood, and regarded them as reaction products.

Escherich and Miyajima (9), in 1911, also found that the disease was highly infectious. These workers contributed some important details to the genesis of the polyhedral bodies within the nuclei of certain tissues. From their experiments, however, they concluded that the polyhedra were the carriers of the virus, although they were unable to place the microörganism within any existing bi-

ological classification. Knoche (18), in 1912, adhered more or less to the above view with the exception that the polyhedra were placed by him among the microsporidian protozoa. According to Knoche little motile elements invade the cytoplasm of the host cells where they multiply. Then invasion of the nucleus occurs, where the elements lose their ameboid movement and transform into the cyst-like polyhedra.

In 1924, Komárek and Breindl (19) concluded that the polyhedra are not the actual parasites, but that the etiological agent resides within them. These workers believe that the bodies are to be compared with plant galls and like them are produced as a specific reaction against the stimulation of the causative principle. They further claimed that at times the virus also exists free and when in this condition will pass through the pores of Berkefeld filters.

Grasserie, Jaundice, or Gelbsucht

The history of grasserie in silkworms is even more diversified than the foregoing account of Wipfelkrankheit.

In 1896, Krassilstschick (20) attributed grasserie to a bacterium, *Micrococcus lardarius*, found in the intestine and in the body cavity of diseased silkworms. Bolle (3, 4), in 1898, discovered polyhedral bodies in grasserie worms. After much work he considered them etiologically important, and placed them among the sporozoa, under the name of *Microsporidium polyedricum*. Marzocchi (21), in 1908, supported Bolle's views.

v. Prowazek (26), in 1907, reported that grasserie material is still infectious after the polyhedral bodies are removed by filtration through several layers of filter paper. For this reason he regarded the polyhedra as reaction or by-products of the disease. The etiological agent, v. Prowazek thought, belongs to a group of protozoa named by him *Chlamydozoa*. Following the fixing and staining technique developed for studies on trachoma, epithelioma of fowls, molluscum contagiosum, and vaccinia, v. Prowazek found in cases of grasserie minute coccoid bodies surrounded by a capsule-like substance. These chlamydozoa were found within the pathological nuclei of silkworm tissue, they also occur in the blood; they are extremely small and can only be seen in preparations colored according to the method of Giemsa. v. Prowazek named the microörganism *Chlamydozoa bombycis*.

Wolff (36), in 1910, supported v. Prowazek in studies on a polyhedral disease of *Bupalus piniarius* L. The Polyederkrankheit of this form, according to Wolff, is a veritable "Chlamydozoonosis" and the etiological agent received the name *Chlamydozoa prowazeki*. Wolff further claimed that bacteria, especially streptococci, played an indirect rôle in precipitating the disease; in other words, bacteria and chlamydozoa live in symbiosis. Wolff also considered the polyhedra to be reaction products.

Sasaki (29) in 1910, stated that grasserie may be due to a variety of initial causes, such as exposure to bacteria or bacterial products, formol, camphor, or to feeding with some food other than mulberry leaves.

v. Prowazek (27), in 1913, gave a further account of his filtration experiences with grasserie material. An emulsion from grasserie cadavers was prepared and passed through an ultrafilter made of a Berkefeld candle covered with agar.

The filtrate contained neither polyhedral bodies nor bacteria, but all silkworms inoculated with it died of grasserie. From these experiments v. Prowazek concluded that in some cases healthy larvae can be infected with diseased material freed from polyhedral bodies. In this paper he gave no further proof for the existence of his chlamydozoa, and, therefore, it seems likely that he was no longer impressed with their etiological importance.

Hayashi and Sako (15), in 1913, came to the conclusion that the silkworm polyhedra are the carriers of the grasserie virus. This view reverts to Bolle's contention of 1898 and also supports Escherich and Miyajima (1911) and Knoche (1912) in their work on Wipfelkrankheit.

Acqua (1), in 1918–19, showed that the grasserie virus is an invisible filterable virus which can be passed through fine Berkefeld candles. This worker confirmed in principle the work of Glaser and Chapman (5, 6, 10, 13, 14), in 1913–18, on wilt, a polyhedral affection of gipsy-moth larvae and pupae.

Aoki and Chigasaki (2) (1921) stated that polyhedral bodies shaken in physiological salt solution and centrifuged more than ten times were just as infectious for silkworms as were the unwashed bodies. The entire work of these authors is devoted to proving that the polyhedra are parasitic.

Paillot (24, 25), in 1924 and 1926, found that grasserie silkworm blood freed from cellular elements and polyhedra by centrifugation and filtration through three layers of filter paper was infectious. He also discovered that he could initiate grasserie by inoculating worms with the blood of animals infected 2 days previously and in which no polyhedra could be detected at that time. Paillot regards the disease to be due to ultramicroscopic intracellular organisms which destroy the nuclei of certain cells. The parasites were first found within the cytoplasm, then within the nucleus where they actively multiplied and later destroyed the cell and passed into the blood plasma. The parasites were invisible by ordinary light, but under the ultramicroscope they were visible as minute animated granules of less than 0.1μ diameter.[1] These granules were only seen in preparations examined in the fresh state and were not found in normal blood; they were arrested by fine-pored porcelain filters, and filtrates obtained under such conditions were not infectious. Porcelain filters having larger pores permitted them to pass, and filtrates procured under these conditions were infectious. Paillot contends that the granules must be etiologically important, since the infectiousness of the filtrates is associated with their presence. Paillot groups the minute particles under a new name, *Borrellina*, in honor of Professor Borrel of the Pasteur Institute.

Wilt

Wilt, a polyhedral disease of gipsy-moth larvae and pupae, was first studied in America by Jones (17) in 1910. This worker found that, after wilted gipsy larvae had been dead for some time, bacteria could be demonstrated without difficulty; but when attempts were made to isolate bacteria from caterpillars that had recently died in the field, the tubes ordinarily remained sterile, and when a growth was obtained the different species of bacteria isolated behaved in a manner similar

[1] By ultramicroscope the dark field is probably meant.

to the non-pathogenic intestinal flora. In this work the polyhedral bodies were apparently not considered important.

Glaser and Chapman (12) in 1912 described a minute, motile coccus (*Girococcus flaccidifex*) as the cause of wilt. This publication was premature and the work was poorly executed, for it was later discovered that nearly all of the animals used for experimentation had become accidentally infected with this bacterium, and that it had simply precipitated the course of the disease which was already present in chronic form. In 1913, Glaser and Chapman (13) corrected this serious mistake and showed that wilt is caused by a filterable virus. The polyhedral bodies were interpreted as reaction products. From 1913 to 1918, these workers (5, 6, 10, 13, 14) verified and extended this work on gipsy moths and also described a number of polyhedral diseases in other species such as army worms, cutworms, cabbage worms, tussock-moth caterpillars, and tent caterpillars. Some recent work (1925–27) on silkworms, gipsy-moth larvae, and tent caterpillars will be included in this chapter.[2]

Histopathology of Polyhedral Diseases

The pathological changes occurring within the tissues of insect larvae suffering from polyhedral disease have been frequently described. The most detailed descriptions, with the technical procedures involved, are those of Conte and Levrat (7), v. Prowazek (26, 27), and Paillot (24, 25) in studies on grasserie of silkworms, of Escherich and Miyajima (9) in their work on Wipfelkrankheit of nun-moth caterpillars, and those of Glaser (10) in work with gipsy-moth larvae. The histopathology of these diseases, as in many diseases of humans and lower animals, is highly important in diagnostic work. For this reason alone, an accurate picture of the changes occurring within the tissues is necessary. The histopathology varies very little in different species. The method of Giemsa gives the best results in the study of tissues and, in view of the fact that dead larvae disintegrate completely, material from living insects must be used for this work. By infecting a large number of healthy individuals with the blood of diseased animals and fixing a specimen every few days, one is able to observe all stages of the disease.

The most striking changes occur within the nuclei of affected cells. If the untreated nuclei of fat or blood cells in early cases of the disease are carefully examined, many minute, violently dancing granules may be seen within. In 1915, similar granules were observed and described, and at that time were regarded as either particles of degenerating nuclear material or minute microörganisms. One finds such tiny granules in

[2] An article entitled, Studies on the polyhedral diseases of insects due to filterable viruses recently appeared in *Ann. Entomol. Soc. Am.*, 1927, xx, 319. The details of recent experiments are described in this article.

fresh films of all polyhedral material either dead or alive. They can be distinguished readily from pigment and other granules by their small size and greater vibration amplitude. Sections of diseased nuclei, prepared according to the method of Giemsa, demonstrate these little granules which stain red, are either single or double, and resemble micrococci. It is difficult to determine definitely the nature of the tiny particles, but by prolonged study of preparations greatly magnified through the use of the cardioid condenser one fails to be impressed with the possibility of their etiologic importance. The following experiment emphasizes this fact. Tissue cultures of normal silkworm fat and blood cells were prepared under sterile conditions on depression slides. Silkworm serum or Ringer's solution was used as a medium. The preparations were held for 2 or 3 weeks, after which time various degrees of cellular degeneration of all the fat and some blood cells were observed. The blood cells situated near the periphery of the coverslip showed growth, often multiplied and formed syncytia. This reaction is produced under the influence of oxygen which filters through the thin layer of vaseline (11). Within the normally multiplying blood cells, vibrating granules were not seen. All of the degenerating fat and blood cells, however, showed minute, vibrating particles within the cytoplasm and nuclei indistinguishable from those seen in grasserie or wilt material. When fixed and stained many of them seem morphologically identical with the granules found in disease. This is not offered as absolute proof that the granules under the different conditions are identical. Nevertheless, it shows that normal cells permitted to degenerate also exhibit minute, violently vibrating granules.

In normal silkworms, gipsy or tent larvae (Plate 2, Fig. 3), nuclei of cells exhibit throughout small round grains of chromatin. The nucleoli are quite large and numerous; according to Paillot they always exceed ten in number within the fat cells of silkworms. Within the cytoplasm, according to this writer, the chondriome (mitochondria collectively) is represented by filaments distributed between the fat vacuoles (Plate 3, Fig. 10).

Sections show that the polyhedra originate within the nuclei of the hypodermal, fat, tracheal matrix, and certain blood cells. They are not found within the nuclei of other cells. The first indication of a diseased cell seems to be represented by a fusion of the chromatin grains and nucleoli. The fusion results in a large, dense, highly chromatophilic mass (Plate 3, Fig. 11) which becomes speckled with minute, refractive bodies arising, according to Paillot, from the chromatin grains. Within

the clear area, left by the condensation of the nuclear material, Giemsa's method demonstrates many minute granules which bear a close resemblance to the dancing particles observed in the fresh preparations previously mentioned. Later, within the clear nuclear zone, the polyhedra arise as very small individuals (Plate 3, Fig. 12). At this stage the borders of the bodies are often faintly outlined by stains. The inclusions increasing in size become more and more refractive, finally do not stain at all, and the nucleus of the cell swells to an enormous size, sometimes measuring three times or more the diameter of a normal one. This hypertrophy is due to the progressive increase in size of the inclusion bodies which fill the entire nucleus (Plate 3, Fig. 13). All polyhedra are in the same stage of development within an individual nucleus, but enormous differences in development are observed when several nuclei are examined. Small polyhedra in the process of formation are somewhat rounder than larger individuals. This phenomenon may be accounted for by the fact that, as the polyhedra grow, they become closely packed and press upon one another thus producing faces and angles. As the bodies increase in size and become more refractive, the little dispersed granules as well as the remains of the chromatin mass disappear, and finally nothing remains but the nuclear membrane enclosing the polyhedra. Sometimes a portion of the chromatin mass persists till the nucleus disintegrates, but most frequently it vanishes before this event. Naturally such profound nuclear changes cause cytoplasmic disturbances, the earliest of which occur within the mitochondria and are characterized by the disintegration of filamentous forms of these structures into dispersed granules. Later, after the nucleus has become completely incapacitated, the entire cell disintegrates and the inclusion bodies escape into the body cavity. It is remarkable to what extent affected nuclei may be injured before disintegration occurs (Plate 2, Figs. 4 and 5). The degeneration of the cells beset with polyhedra is probably independent of, although coexistent with, the lysis of all the tissues mentioned previously. Shortly after death all tissues disintegrate, but it is difficult until the last to detect any striking changes within the cells of the muscles, intestinal epithelium, Malpighian tubes, salivary and other glands, ganglia, nerves, and gonads.

From evidence obtained by means of these studies and others to be presented later, it seems that one is justified in regarding the polyhedral inclusions as reaction bodies resulting from special nuclear changes produced by the virus.

Normal and Pathological Blood Cells

The blood has been extensively used by various workers in determining whether an insect is suffering from polyhedral disease, and if so, how far the condition has progressed. If large numbers of healthy insects of a particular species can be reared, blood examinations are not necessary. In our work with silkworms we were able to dispense with the so-called blood test, due to the fact that we have been able to keep our stock entirely free from grasserie for 5 years. If one collects living nun- or gipsy-moth larvae from the field for use in experimental work, the examination of the blood for polyhedral bodies is absolutely necessary, since so many chronic cases of Wipfelkrankheit and wilt exist, which may assume an acute form at any time. The appearance of spontaneous cases among controls resulting in the ruin of well-executed experiments is an old difficulty, well-known to European and American workers. Escherich and Miyajima (9), in 1911, first made use of blood examinations in selecting healthy nun-moth larvae. The blood from each individual was examined two or three times, permitting an interval of several days to elapse between successive examinations. If at the last examination no polyhedral bodies were found within the plasma or within the nuclei of certain blood cells, the animals were pronounced healthy and were considered fit subjects for experimentation. Escherich and Miyajima found that the blood of nun-moth larvae is a reliable index of conditions existing within the nuclei of other tissues, and Glaser and Chapman in 1913 used the blood test to advantage in their work on gipsy-moth wilt. The test works very well but requires a great deal of time. Therefore, it is preferable to acquire by careful breeding and selection a stock of animals entirely free from disease. This can be accomplished also by gathering a sufficient number of larvae from lightly infested districts. In dealing with some species of insects, however, it seems necessary to resort to blood examinations, and for this reason a knowledge of normal and pathological blood is essential.

Much work on the blood cells of a variety of insects has been done, but it is best to confine the discussion to silkworms, gipsy-moth larvae, and tent caterpillars. Some cells of these forms examined in the fresh state show pseudopods and slow independent progression. The same specimens can be fixed later in methyl alcohol, stained with Harris' or Delafield's hematoxylin, carefully dehydrated, cleared, and mounted. The ordinary blood-staining methods used in hematology of vertebrates do not give good results consistently, although at times one obtains fairly

satisfactory pictures with the methods of Giemsa, Romanowsky, and Wright.

The classification used by Paillot (23), and by Metalnikow and Gaschen (22) for insect blood cells may be followed. The most dominant type of cells found (approximately 40–50 per cent) comprises the real leucocytes or phagocytes (macronucleocytes of Paillot) which engulf bacteria and other foreign bodies. These cells are extensive in size each having a large nucleus and a considerable amount of clear cytoplasm. They exhibit active ameboid movement and pseudopodial processes (Plate 3, Figs. 14 and 15).

The next most common type of cells (approximately 25–30 per cent) is the proleucocytes (micronucleocytes of Paillot). They are generally somewhat smaller than the foregoing, are rounded or oblong, and never show any ameboid movement. The nucleus is quite small in comparison with that of the phagocytes and the cytoplasm appears to be denser. They often contain large granules resembling the eosinophilic granules in higher animals.

In silkworms the lymphocytes rank next numerically (approximately 10–15 per cent). They are rounded elements each with a large nucleus and a small amount of granular cytoplasm and never show independent movement. Fig. 16, Plate 3, represents the spherule or mulberry corpuscle. This type of cell is not very common in silkworms and gipsy-moth larvae, ranking third or fourth numerically (approximately 10–15 per cent). In tent larvae they are exceedingly common, however, ranking second in abundance (approximately 25–30 per cent). They can be distinguished readily from other blood cells by the addition of a little neutral red to the preparation which produces a picture showing curious, irregularly rounded cells with a peripheral zone of spaces filled with red-staining spherules. The same cells are often encountered with empty spaces. The nucleus is large and centrally located. On crushing spherule cells with filled vacuoles, one obtains a picture of scattered spheres and distorted and elongated nuclei. The spherule cells have no independent movement. The cytoplasmic spherules have often been mistaken for polyhedra in pathological cells.

The pseudo-oenocyte corpuscle represents a type of cell not common, but which probably ranks fourth or fifth numerically in silkworms and tent caterpillars. It is a non-motile, large, rounded or oblong cell with a dense, homogeneous cytoplasm and a small nucleus. It has a light yellow tint in the fresh state, and superficially resembles the stationary, large, glandular cells (true oenocytes) found around the spiracles. Lastly, at times, giant cells are encountered. These are found in the blood of perfectly normal animals and are simply huge cells containing five, six, or more nuclei. Some degenerating forms of unknown cells are also often found in normal blood. These at times have little cytoplasm remaining, and the particles within them show violent Brownian movement.

In diseased larvae polyhedra develop within the nuclei of the phagocytes, proleucocytes, and lymphocytes, cells which are considered by some workers to represent stages in the development of the same element. The inclusions are never found within the nuclei of mulberry corpuscles, oenocyte-like cells, or giant cells. When a few small refractive bodies

are found within the nucleus of a phagocyte it is rather difficult to decide whether infection has occurred or not. Such pictures have been seen, but the larvae subsequently failed to develop symptoms. When such a nucleus is encountered as represented in Fig. 17, Plate 3, however, there can be no doubt that the insect will sooner or later succumb. As is the case within the other tissues, the polyhedra develop within the nuclei of the blood cells; when found in the cytoplasm, they have been phagocytized. The origin of the inclusions in the blood cells is also preceded by a concentration of the nuclear substance and the formation of a dense central mass. Around this, one finds a zone in which appear refractive granules that gradually develop into polyhedra and later completely fill the nucleus in the manner outlined for other tissues.

During the last stages of the disease, when the tissues and blood cells begin to disintegrate, myriads of polyhedra are found floating free in the blood plasma. These free polyhedra, together with the liberated fat drops from the degenerating fat body, impart to the blood of heavily diseased silkworms a yellowish milky appearance. From the outline just given, it is evident that the blood is a fairly reliable index of the condition of a particular larva, and if two blood examinations are made on each animal with an interval of a few days between the tests, one can be fairly certain whether polyhedral disease exists or not.

Predisposing Causes

Insofar as is known, the race of a particular insect has very little to do towards predisposing it to polyhedral disease. It seems that the monavoltine, bivoltine, and trivoltine races of silkworms are all equally susceptible. Work on gipsy moths has also shown that *Porthetria dispar* and the race *japonica* are susceptible to wilt to the same extent. The sex of a particular insect has no effect; age, however, has some influence on susceptibility. All of the larval stages are susceptible to polyhedral disease, but it is more easily transmitted to the old than to the young larvae. Young pupae acquire the disease readily, but after the adult moth tissues are already formed it seems impossible to infect them. The adults, or moths, are refractory and no evidence exists to prove that eggs are killed by polyhedral disease.

Temperature seems important, but to what extent this is separable from humidity is not known. At any rate, insects that are held for a prolonged time at an unusually high temperature (90–95°F.) are much more easily infected than those held at cooler temperatures. The incubation period of the disease is also appreciably shortened by exposing the

animals to high temperatures. Starvation or poor food also seems to break down the resistance to infection and shortens the incubation period. Years ago Escherich and Miyajima, v. Prowazek, Glaser, and others showed that resistance or susceptibility to polyhedral disease after infection depends intimately upon temperature, starvation, quality of food, and other environmental conditions; in other words, these factors may convert chronic cases, which often survive, into acute ones, which invariably die.

Exciting Cause

Healthy silkworms can be infected by inoculating or by feeding them grasserie blood taken from living diseased individuals. The same result can be obtained by using an emulsion prepared from any tissue excised from diseased worms or by using the body liquids from dead individuals. When the animals are so treated they assume the jaundiced appearance in from 4 days to 1 week and die of grasserie in approximately from 5 to 10 days. Sometimes death does not occur until 14 to 18 days, and the time is somewhat longer following infection by feeding than that following direct inoculation.

With gipsy-moth larvae and their wilt incitant or tent caterpillars and their virus, approximately the same result is obtained, with the exception that the time from infection by feeding to death is somewhat longer, on an average of 21 days.

From such a series of infected larvae another series of healthy animals can be infected and the disease will again appear within the required time. The blood or other materials from these is again infectious for another healthy series. One can thus continue indefinitely to infect larvae. Clearly, the pathogenic agent multiplies and increases in amount within infected insects. The infectious blood or other materials from diseased worms, however, do not reveal microscopically and culturally any bacteria or other microörganisms with which one can associate the disease. Indeed, in general, nothing is found excepting polyhedral bodies, tissue cells, and cellular débris. Much bacteriological and microscopical work has been done on infectious polyhedral material and from an etiological standpoint may be termed entirely negative. The virus is apparently not biologically organized as a cell. Under the dark field with high magnifications, myriads of minute dancing particles are observed in polyhedral material, but these are interpreted as protein particles of no etiological importance. Similar particles are found in normal larval blood and tissues when they are removed and held for several days to permit normal cellular disintegration to occur.

Infectious blood greatly diluted (1:1,000,000) still retains its infectivity and produces disease when inoculated into healthy insects. The virus in diluted blood or in diluted emulsified cadavers passes through Berkefeld filters and retains its infectivity. The polyhedral viruses tested (grasserie, gipsy-moth and tent wilt) are easily filterable through Berkefeld V and N candles. They can be filtered through W candles with difficulty, but cannot be passed through Pasteur-Chamberland filters. The results may depend upon the size or formation of the filter pores, upon the flexibility or rigidity of the virus particles, upon the electrical charge, or upon other factors.[3] All the filtrations were performed at a pressure of 74 mm., and other factors such as time of filtration, dilution, etc., were maintained constant. The infectious filtrates are free from polyhedral bodies, bacteria, or other formed bodies which might be considered etiologically important. Paillot has recently (1926) been able to verify most of these findings. His interpretation in regard to the nature of the etiological agent is somewhat different, however, as is explained in the historical section.

Experiments with Washed and Fragmented Polyhedra

Certain workers have emphasized the fact that the polyhedral bodies are of great diagnostic value but are not etiologically related to the disease. This opinion was based on results of certain filtration experiments, on the microscopical appearance of the bodies, on their staining reactions, and on their physical and chemical nature. However, the work of Aoki and Chigasaki (2) and of Komárek and Breindl (19) again enveloped the above opinion in doubt. As previously mentioned, the two Japanese workers attempted to prove that the polyhedra are parasitic. Komárek and Breindl claimed that the virus resides within these bodies and for this reason further experiments were undertaken by the author.

At separate times, two lots of polyhedral material were prepared; one was derived from a large number of tent-wilt cadavers; the other from silkworm-grasserie cadavers. All of the larvae used had been dead only 3 days and on death were immediately placed on ice to inhibit development of bacteria. It is well known that the intestinal flora of herbivorous larvae comprises few organisms unless a bacterial disease prevails. In each case, after enough material had

[3] The classification of filters is the one given in Arthur H. Thomas' catalogue, Philadelphia, 1926. *Berkefeld filters:* "W" fine; "N" medium; "V" coarse. *Pasteur-Chamberland filters:* "F" coarse texture and suitable for ordinary filtrations; "B" fine and suitable only for pressure.

accumulated, the dead larvae were crushed in a mortar with physiological salt solution. The emulsion was then passed through cheesecloth, diluted a little more, and drawn through a hard, fine-pored paper (Whatman No. 50) by means of suction. The filtrate which abounded in cellular débris and polyhedra was then washed with physiological salt solution and centrifuged repeatedly, 20 times in the case of the tent and 22 times in the case of the silkworm material. Care was taken to thoroughly wash the tubes between each centrifugation. A clean pipette was used in stirring up the sediment during each washing. This process extended over a period of approximately 5 days, and during the interval between each cleansing, the sediment was held on ice. After this, when it was thought that most of the virus had been removed, the final sediment obtained had the gross appearance of white chalk, and on microscopic examination consisted almost entirely of polyhedral bodies with the exception of a few urate and other crystals. The sediment, which in each case amounted to about 1 gm. of polyhedra, was shaken up with 5 cc. of physiological salt solution. 0.1 cc. of this milky white liquid was injected into each of 20 larvae. Twenty tent larvae were injected with the wilt-polyhedra suspension, and 20 silkworms with the grasserie-polyhedra suspension. Of the 40 larvae so infected, 7 died of a bacterial septicemia and 33 transformed into moths. The blood of all inoculated worms was examined twice during the course of the experiment, at an interval of 1 week, but polyhedra were never seen in the plasma nor within the nuclei of the cells.

The above experiment was repeated the following year with the exception that pains were taken to fragment most of the polyhedra by grinding in a mortar. This process was followed microscopically. Further fragmentation can be obtained by pressing them between panes of glass. 20 tent larvae and 20 silkworms were again injected with their respective polyhedra. 13 died of bacterial septicemia, and 27 transformed into moths. Blood examinations were also entirely negative. It has also been recently found that no disease is produced when cleaned grasserie polyhedra or wilt polyhedra are fed to healthy silkworms and tent caterpillars respectively. It seems, therefore, that the experiments described taken in conjunction with the filtration experiments aid in eliminating the possibility that the polyhedra are parasitic or that they enclose a parasite.

Host Specificity of the Viruses

In 1915, it was pointed out that, although the gipsy moth reached this country in 1869 and became a serious pest in 1889, wilt among the larvae was unknown prior to 1900. It can be stated safely that during a period of 11 years, before active suppression work by the state of Massachusetts was stopped and before the Federal Government intervened, not a single case of wilt was seen or recorded, although entomologists and field men were continually on the lookout for parasites and diseases. At

that time it was known that wilt produced epidemics among gipsy moths in the Old World and Wipfelkrankheit, a similar disease among nun-moth larvae in Europe, had been recognized before 1892 when v. Tubeuf Tangl, and Wachtl and Kornauth made valuable contributions to the subject. When wilt appeared in this country in epidemic form after 1900, entomologists and others began to wonder how the gipsy moth, heretofore an extraordinarily healthy insect, acquired such a serious disease which proved of tremendous aid in the natural control of the pest. At that time it was recognized that a wilt occurred among some of our native American forms, especially among the apple tree tent caterpillars. The theory was advanced that tent wilt and gipsy wilt were identical, that the race of gipsy moths accidentally imported into America were at first immune, and that this immunity was gradually lost. Another theory assumed that the European Wipfelkrankheit and wilt were identical and that the former disease was imported into America after 1900 through the importation of trees and shrubs. A third theory assumed that all the polyhedral diseases were different, and that gipsy wilt was imported from European gipsy moths with plant shipments or with some of the insect parasites sent over from Europe by the Federal Government after 1905.

The only experimental proof offered on this question of specificity is Bolle's claim that he succeeded in infecting healthy nun-moth larvae with jaundiced silkworm blood. Much work has recently been done to see whether some of these diseases, so similar superficially, are due to the same etiological agent. Three viruses were used in this work, namely, the gipsy-moth-wilt virus, the tent-wilt virus, and the silkworm-grasserie virus. A large series of gipsy-moth larvae was inoculated with infectious tent-caterpillar-wilt blood. Another series were inoculated with infectious silkworm-grasserie blood. The experiment was then repeated by feeding gipsy larvae with the diseased blood of the other two species. No disease resulted. In an identical manner experiments were performed to see if tent caterpillars were susceptible to gipsy-moth wilt and silkworm grasserie; and, lastly, silkworms were tested with gipsy-moth and tent-caterpillar wilt. No disease resulted in any of these cases.

These experiments show, therefore, that the grasserie, tent-wilt and gipsy-wilt viruses are distinct and specifically limited to their hosts. Bolle's moth larvae must have been infected with Wipfelkrankheit prior to the inoculation with the grasserie material.

It also seemed important to determine whether the polyhedral dis-

eases were related to sacbrood, the virus affection of bee larvae. In 1916, Chapman and Glaser inoculated gipsy moths with sacbrood virus obtained from Dr. G. F. White, with the result that no disease of any sort developed. Dr. White performed the reciprocal experiment and inoculated bee larvae with gipsy-wilt virus. The bees were not affected.

Immunity and Transmission of Virus through Eggs

Experimenters have often noticed that a certain number of infected larvae do not develop polyhedral infection and do transform into moths. In 1915, this immunity was observed in the gipsy-moth experiments and it has also been encountered many times in the silkworm-grasserie work. Attention was also called to the same fact in field studies on gipsy-moth wilt. During a severe epidemic of wilt, in a heavily infested wood lot, thousands of gipsy larvae die daily of the disease; and the body fluids everywhere soil the leaves which are eaten by others. Congregation in immense masses on the trees occurs prior to pupation, and in such places the mortality is tremendous. An enormous reduction in the number of adult moths, and consequently in the number of egg clusters deposited will occur, but a complete extermination is never possible owing to the immunity of certain individuals in each generation.

Bolle reported finding polyhedral bodies in silkworm moths. Wahl not only found them in newly hatched nun-moth larvae, but also in pupae and moths. Escherich and Miyajima, on the other hand, were unable to find the bodies in adult nun moths. Recently Rebouillon (28) and Paillot reported that they were able to detect grasserie in silkworm moths and Paillot can even detect infection within eggs. If true, this would constitute an extremely valuable discovery for the silk industry, because infected moths and eggs could be readily separated from the non-infected ones much after the very practical scheme devised by Pasteur for pébrine. Glaser and Chapman, however, repeatedly examined moths and eggs obtained from experimentally infected larvae without finding evidences of polyhedral disease. During field epidemics many female gipsy moths were dissected and examined for the presence of polyhedral bodies, but such examinations were invariably negative. Recently tent and silkworm moths and eggs were also subjected to examinations, but no positive evidence was obtained in this manner.

Nevertheless, much indirect evidence exists which seems to point to the fact that polyhedral viruses survive in moths and are transmitted through the eggs to the succeeding generation. Entomologists find that

polyhedral disease never appears severe during the first year of a heavy new moth infestation. Wilt and Wipfelkrankheit only become epidemic after the second and third years. It does not seem likely that increase in virulence due to successive passages can account for this, because the number of passages possible in short-lived, one-generation-a-year animals is small. Furthermore, approximately 8 months intervene between each generation during which time no passages are possible inasmuch as the insect is in the egg stage. Other factors may account for the rapid increase in the extent of epidemics during succeeding years, but one plausible explanation offered is that each surviving but infected moth passes the disease to a portion of its progeny.

Recently some experimental evidence has been obtained in favor of the above contention. Eggs were obtained from tent moths that survived experimental larval infections with tent-wilt virus. Before death, or shortly after, all of the moths were examined microscopically for polyhedra or other evidences of wilt. These examinations were negative. The next spring the egg clusters were "broken up" in order to separate the eggs which are held together by a mucilagenous substance. Twenty-five eggs were then submerged for 10 minutes in a mixture of equal parts of 1:1000 corrosive sublimate and 95 per cent alcohol. This was done to kill any virus that might have adhered to the outside of the chorion. Experience has shown that the wilt virus is rapidly destroyed by this treatment. The eggs were then washed in sterile water and placed on sterile filter paper in sterile glass phials, one egg to a phial. The phials were corked with cotton. On hatching each larva received cherry leaves selected from a region entirely free from tent larvae. The leaves were further thoroughly washed before being tendered. After growing, each larva was transferred to a larger sterile bottle. Every possible precaution was taken to keep wilt infection from these larvae, such as keeping them in a unit in which wilt had never occurred, and keeping away attendants who had been in contact with wilt. In spite of these precautions, three out of 25 larvae so handled independently contracted wilt and died in the fourth and fifth stages. The others completed their development. Naturally, if all had been confined together, the mortality would have been higher and some would undoubtedly have become infected and survived. Thus, it is easily seen how a progression might be obtained with each succeeding generation. From the experiment, however, the only statement one is justified in making is that it seems likely that the disease is transmitted from generation to generation through the eggs of a certain proportion of individuals.

The Resistance of the Polyhedral Viruses to Physical and Chemical Agents

Little work has been done on this phase of the subject. A few facts, however, are worth recording. Grasserie or wilt blood when held at 60°C. for from 15 to 20 minutes loses its virulence. Grasserie or wilt blood when dried at room temperature remains infectious for at least 2 years. Bolle states that after drying grasserie blood on glass slides and keeping it for 1 year he was able to induce the disease in silkworms. Wahl reports that the virus of Wipfelkrankheit is virulent under the same conditions after 3 years. Escherich and Miyajima report that Wipfelkrankheit blood will resist glycerol for 5 days. Wilt and grasserie blood have been kept in 98 per cent glycerol for 6 months without losing its virulence. Escherich and Miyajima report that the Wipfelkrankheit virus withstands putrefaction. Wilt blood resists putrefaction for an indefinite time. Sufficient data concerning the effect of putrefaction on the grasserie virus do not as yet exist. Wilt and grasserie viruses are inactivated by exposure for 9 hours to sunlight. When wilt or grasserie blood is dried and submerged in 80 per cent alcohol for 15 minutes it loses its activity. Wilt blood dried and submerged in 5 per cent carbolic acid loses its infectiousness in approximately 3 weeks.

Control of Polyhedral Viruses

Grasserie or jaundice of silkworms, wilt, Wipfelkrankheit, and the other polyhedral diseases are insidious and extremely infectious. Healthy larvae are rapidly infected by eating food soiled with the juices and tissues of disintegrated cadavers. Feces of diseased living individuals are also dangerous. If polyhedral disease is suspected in a stock from the results of occasional blood examinations, or from the external appearance of the larvae, or if it is evident through the death of but a single individual, the whole lot among which the case or cases appeared should be removed, killed, and burned. The containers should be destroyed or sterilized. For this reason, it is best to divide the entire stock into as many small groups as is practicable. By so doing, if polyhedral disease, or any other disease for that matter, appears in individual lots it will not spread to the entire stock. Under no conditions should eggs be hatched that were derived from moths that survived larval infection.

Care must be taken not to introduce the disease from the outside through the food or through the purchase of foreign eggs of unknown history. Predisposing causes must be avoided, such as excessively high

temperatures, starvation, and poor or dirty food. Every conceivable bacteriological precaution should be taken in rearing larvae for experimental purposes. Insofar as the cultivation of silkworms in mass is concerned—for industrial purposes—some of these suggestions may not seem practical. With certain modifications, however, to meet industrial conditions, most of these precautions can be applied.

The Economic Value of Polyhedral Diseases in the Control of Certain Pests

No one who has seen extensive epidemics of wilt or Wipfelkrankheit can fail to be impressed with the tremendous value of these diseases. They accomplish a greater reduction of the pests than do the combined efforts of all of their hymenopterous and dipterous parasites superimposed upon human spraying and other control measures. In this country in combating the gipsy moth, insect parasites have proven valuable and roadside spraying of forest trees is also of aid. Parasites, however, do not increase as rapidly as does the wilt, and in dense woods, where spraying hoses will not reach, the wilt, nevertheless, takes a tremendous yearly toll. The same is true in Europe among the gipsy and nun moths. v. Tubeuf, Wahl, Escherich, and others have many times emphasized the great importance of the polyhedral diseases in the control of these insects.

Can we aid the spread of these diseases or increase their efficiency? The experiment has been tried several times in different ways, but the results did not withstand critical analysis. Polyhedral disease already exists wherever the pests are plentiful. Therefore, if more virus is introduced no conclusions can be drawn because controls fail. In the spring a section of a forest was sprayed here and there with emulsified cadavers from the previous year. Later in the season wilt took just as great a toll in another untreated lot of the same size which was miles away from the first, and which was approximately as heavily infested with caterpillars as was the treated lot. Perhaps a more accurate experiment would give quantitative results that would show that the distribution of the virus in the first lot accomplished much. Quantitative field experiments among high trees and underbrush subject to heat, cold, and torrential rains are more difficult to perform than experiments in a laboratory under conditions that can be controlled. However, some sort of human endeavor combined with the natural efficiency of the polyhedral diseases still offers some hope for the control of these pests.

SUMMARY

1. The larval and pupal stages of certain insects of the order Lepidoptera are susceptible to polyhedral diseases. These maladies are distributed throughout the world and affect some of our worst agricultural and forest pests often producing huge epidemics among them and hence contribute inestimably towards their natural control. Unfortunately, the silkworm is also susceptible to one of these diseases. In America the polyhedral diseases of the gipsy moth, tent caterpillar, army worm, and other species is commonly termed "wilt." In Europe a similar disease of nun-moth larvae is called "Wipfelkrankheit." When appearing in silkworms it is known as "grasserie," "jaundice," "Gelbsucht," or "Fettsucht."

2. The literature on polyhedral diseases is voluminous and opinions as to their nature and etiology are interesting and extremely diversified.

3. The diseases are highly infectious and are easily induced by inoculating or feeding diseased blood or liquefied cadavers to healthy larvae. The exciting agents are viruses that are still infectious after being greatly diluted, and that are easily filterable through Berkefeld V and N candles. They pass through W candles with difficulty, and cannot traverse Pasteur-Chamberland filters.

4. Infectious filtrates are free from bacteria, polyhedral bodies, and other visible agents that might be deemed etiologically important.

5. Under the dark-field illumination with high magnification minute, dancing granules are observed in the filtrates. Many similar granules are often seen free in the lymph of diseased larvae, and within the cytoplasm and nucleus of affected cells. These particles are considered to be products of cellular disintegration.

6. Infection occurs naturally by way of the mouth.

7. The period from infection to death varies from 5 to 18 days, with an average of 10 days. This variation depends upon the method of administration (feeding or direct inoculation), upon the natural resistance of the host, upon the concentration of the virus, upon temperature, and other factors.

8. Polyhedral bodies vary in regard to size and shape in different species of host and also within individual hosts of the same species.

9. The polyhedral bodies can be freed from active virus by repeated washing and centrifuging. The bodies so treated, entire or fragmented, are incapable of producing disease when injected into larvae or when fed to them.

10. The polyhedra were subjected to certain microscopical, physical, and chemical tests from which no evidence was obtained that might lead one to interpret these structures as living entities or as enveloping living material.

11. The polyhedra originate within nuclei of the hypodermal, fat, tracheal matrix, and certain blood cells.

12. The blood is a useful diagnostic index and can be used in following the progress of the disease.

13. From evidence obtained it seems that one is justified in regarding the polyhedra as reaction bodies arising in certain cells in the form of by-products of nuclear changes produced by the virus.

14. Silkworm grasserie, tent-caterpillar wilt and gipsy-moth wilt, though similar histopathologically, are nevertheless caused by different viruses.

15. Some larvae are immune to their specific virus.

16. Evidences of polyhedral disease cannot be detected in moths and eggs, but an experimental result is presented which seems to demonstrate that the tent virus is transmitted from generation to generation through eggs.

17. The polyhedral diseases are of economic value in the control of certain pests and may possibly be utilized more advantageously in the future.

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EXPLANATION OF PLATES

Plate 1

Photograph showing wilted gipsy-moth caterpillars hanging to the trunk of a tree.

Plate 2

Photomicrographs. × 540.

Fig. 1. Nun-moth polyhedra.

Fig. 2. Silkworm polyhedra.

Fig. 3. Fat cells of a normal gipsy-moth larva.

Fig. 4. Fat tissue from a grasserie silkworm, showing nuclear lesions and polyhedral bodies.

Fig. 5. Fat tissue from a wilted gipsy-moth larva, showing nuclear lesions and polyhedral bodies.

Plate 3

Sketches.

Fig. 1. Polyhedral bodies from a case of wilt in a gipsy-moth larva.

Fig. 2. A common type of silkworm polyhedron.

Fig. 3. A double polyhedral body from gipsy-moth wilt.

Figs. 4–9. Polyhedral bodies cracking and fragmenting.

Fig. 10. Fat cell of a normal silkworm; nucleus with chromatin grains and numerous nucleoli; filamentous mitochondria in cytoplasm.

Fig. 11. Pathological condition in a fat cell of silkworm; condensation of chromatic material and clear zone which contains scattered granules; the mitochondria in cytoplasm have fragmented.

Fig. 12. Pathological fat cell of silkworm showing remains of chromatin lump and formation of small polyhedral bodies within the nucleus.

Fig. 13. Pathological fat cell of silkworm showing hypertrophied nucleus filled with fully formed polyhedral bodies.

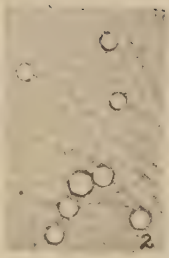
Figs. 14 and 15. Normal leucocytes or phagocytes from silkworm blood.

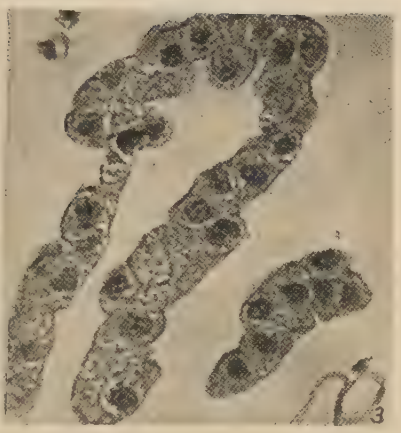
Fig. 16. Normal mulberry corpuscle from silkworm blood.

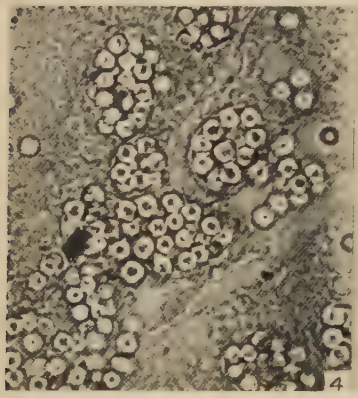
Fig. 17. Pathological silkworm leucocyte, showing hypertrophied nucleus filled with fully formed polyhedral bodies.

PLATE 1

PLATE 2







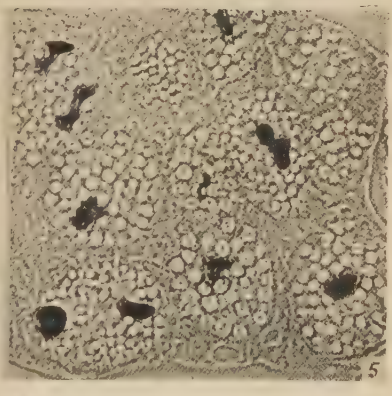
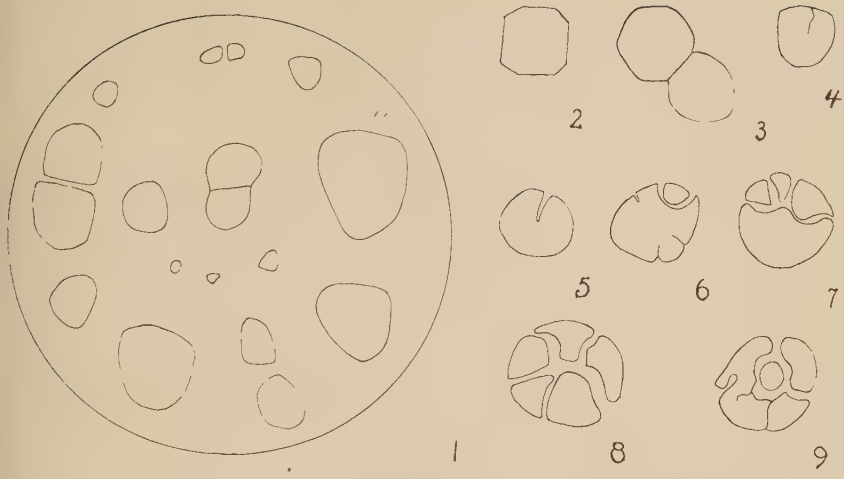
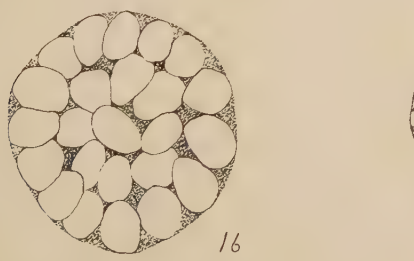


PLATE 3





VIRUS DISEASES OF PLANTS

LOUIS O. KUNKEL

CHAPTER IX

VIRUS DISEASES OF PLANTS

INTRODUCTION

The discovery of filterable viruses has led to the recognition of what might be termed the fifth group of infectious plant diseases. Four groups of diseases are known to be produced by four different kinds of parasitic organisms. They are due to fungi, bacteria, myxomycetes, and protozoa. We do not yet know what causes the diseases in the fifth group but for convenience we say they are due to viruses.

An attempt will be made here to discuss briefly some of the things that are known and some of the things that are not known about virus diseases of plants. Such a discussion may serve to call attention to the similarities as well as to the differences between these diseases and the virus diseases of animals. A consideration of the most important characteristics of the virus diseases of plants may be of some value in suggesting further lines of attack on these maladies and on the related diseases of animals.

The virus diseases that attack cultivated plants are undoubtedly of greater economic importance now than they were 20 years ago though we have no reason to doubt that most, if not all, of them have been in existence for a very long time. Until comparatively recent years they were prevalent over small areas. During the past 50 years due to increased travel and commerce many serious virus diseases have been widely distributed. Unless quarantine measures are carefully enforced, they may become world-wide in distribution. The danger from their spread is all the more disturbing since no one knows how many of them are prevalent in different parts of the world. We know that at the present time the United States are free of a number of virus diseases that attack economic plants. The streak, Fiji and sereh diseases of sugar cane, the corn mosaic transmitted by the corn leafhopper, the rosette disease of peanuts, and the mosaic disease of rice are some of the maladies of the virus group that have not yet reached this country.

Most of our cultivated crop plants are known to be attacked by one or more virus diseases. There are a few, however, that, so far as known, have escaped the diseases of this group. Some of these are asparagus,

cabbage, flax, oats, and pineapples. Virus diseases are more common on herbaceous than on woody plants. Most forest trees are free of them.

Some virus diseases cause heavy losses. Others do little harm and there are a few that add value to the plants they attack. In 1924 the yield of the sugar-beet crop in the Yakima valley in the State of Washington was reduced to one-fourth of that of a normal crop by the curly-top disease of beets (21). Peach yellows causes each year the loss of many fine trees in orchards in the northeastern part of the United States. Virus diseases of the potato cause heavy annual losses in most of the important potato-growing regions of this and other countries. Cucumber mosaic has ruined the cucumber-growing industry in some parts of the United States. Grass mosaic causes or has in the past caused severe losses in many of the sugar-cane-growing countries of the world. The leafhopper-transmitted corn mosaic of Hawaii sometimes causes a total loss in badly diseased fields. On the other hand, grass mosaic on corn in the southern states does little damage (91) and this same disease on certain tolerant varieties of sugar cane causes a very small reduction in the yield of sugar. On certain varieties the mild mosaic of potato is not a serious disease. The yellow color of foliage caused by abutilon mosaic makes *Abutilon thompsoni* a valuable ornamental plant. The infectious chlorosis of *Fraxinus pubescens* gives ornamental value to the aucubifolia variety of this tree (13). The mosaic disease of tulips produces some of the most highly prized varieties of this plant. The much valued Rembrandt variety consists of diseased specimens of the variety Princess Elizabeth.

The virus diseases of plants seldom cause 100 per cent infection of field crops. Even in the worst cases of spread there are usually some plants that escape. Certain influences tend to hold these diseases in check. Most viruses are not transmitted through seed and each new crop from seed is therefore disease-free. They frequently reduce the yield of vegetative parts used in propagation. Streak, leafroll, and the different mosaic diseases of the potato reduce the yield of tubers to such an extent that these diseases might eventually be lost if they did not spread to healthy stocks. Diseased plants sometimes recover. Shaded specimens of *Abutilon thompsoni* were observed by Baur to recover from mosaic disease (12). Robbins reports the recovery of sugar beets from mosaic (74). Sugar-cane plants were found to recover from the streak disease of corn (92) and in certain varieties of sugar cane recovery from grass mosaic is not uncommon (54). Recovery by the sugar-cane variety, D-1135, is so common that mosaic is of little consequence in this variety.

ffected plants sometimes die prematurely. This helps to free crops of isease.

Opposed to these natural tendencies toward a reduction in the prevlence of virus diseases in any crop are certain factors that promote heir increase. The most important of these are the agencies that spread isease to healthy plants. The mosaic of tobacco is sometimes spread y workmen who use tobacco (97). Pickle pickers spread cucumber nosaic from vine to vine (26). Spindle tuber of the potato is transitted by cutting knives used in preparing seed pieces for planting 37). Peach yellows is known to be spread by budding diseased tissue n healthy stocks (87). Swarms of insects belonging to many different pecies spread these diseases far and wide.

A number of different methods have been devised for controlling irus diseases. Plant quarantines delay or prevent their spread from ountry to country. The use of healthy stocks in planting crops that re propagated vegetatively is a valuable control measure. Obtaining tocks from fields that are almost or quite disease-free, seed certification nd the growing of seed plots isolated from diseased fields are measures hat help to keep them in check. Care in preventing mechanical transnission, eradication of weed hosts, roguing out of diseased plants, rotaion of crops, spraying for the control of insect vectors, and alteration of ultural procedures are some of the control methods now in use. But he most satisfactory method of control is to find disease-tolerant, isease-resistant, or disease-immune varieties of the crops affected.

Virus Diseases a Distinct Group

The symptoms produced by virus diseases of plants are enough alike nd are now well enough known to make possible the classification of any ew virus disease with a considerable degree of certainty even before it known that the disease is transmissible or that it is caused by a filterassing agent. In this respect the virus diseases of plants may differ rom those of animals. A few examples will serve to illustrate what is neant. The agent causing the mosaic of *Abutilon thompsoni*, one of the est known virus diseases of plants, has not been shown to pass bacteriaroof filters and has not been transmitted except by grafting. But no ne doubts that this disease belongs with other mosaic diseases of plants. 'o ato mosaic and the curly-top of beets are recognized as virus disases though the agents causing them have not been shown to be filterassers. Both of these maladies were placed in the virus disease group ng before they were mechanically transmitted. No bacterium, fungus,

or other known plant parasite, has been shown to cause symptoms similar to those associated with mosaic diseases or with any of the other well-known virus diseases of plants. It is of course true that not all virus diseases are readily classified by their macroscopic symptoms. It is also true that bacteria and fungi produce in plants some of the symptoms associated with virus diseases. But they do not cause the same set of symptoms or the same general effects. Other characteristics of the virus diseases place them in a distinct group quite as much as does the fact that they are infectious and are caused, so far as known, by agents that are filterable. Many virus diseases are not highly infectious and most of them have not been shown to be caused by filter-passers. That many plant viruses have not been proved to be filterable is not due to a lack of interest on the part of plant pathologists in their filterability. It is easy to determine whether or not a virus is filterable, if it is highly infectious and readily transmitted by mechanical means as are many virus diseases of animals. But a virus that is difficult to transmit mechanically or one that cannot be transmitted except by grafting or by the bite of an insect does not furnish suitable material for filtration experiments. It is not surprising, therefore, that although infectiousness and filterability are the characteristics by which tobacco mosaic was first recognized as a new type of plant disease, these characteristics have been given less attention by plant pathologists than by students of the virus diseases of animals. Animal pathologists have emphasized the fact that the virus diseases of animals are caused by filterable agents. I wish to emphasize the fact that while many plant diseases are caused by viruses known to be filterable many others are caused by viruses that have not, and in some cases apparently cannot, be shown to be filterable. Such maladies must, nevertheless, be included in the virus group of plant diseases because they show characteristics which place them unmistakably in this group.

Symptoms and Other Characteristics

The virus diseases of plants produce three general types of symptoms which appear either alone or in different combinations. The most common of these is chlorosis. So conspicuous is this symptom that plant virus diseases have frequently been referred to as infectious chloroses (11). In the mosaic type of chlorosis, light green colored masses of tissue of varying sizes and shapes are irregularly distributed in, and more or less sharply separated from, the normal appearing green tissues.

In the yellows type of disease, chlorosis is general throughout the affected parts. Deep green colored tissues are not present.

The second type of symptom is necrosis. Groups of cells in certain parts of diseased plants die and collapse. Necrosis occurs in both stem and leaf tissues. It is not confined to chlorophyll-bearing parts as is chlorosis. It is a symptom of a number of different mosaic and yellows diseases. So important is it in the case of leafroll of potato that this disease is referred to by some workers as phloëm necrosis (6). It is the most common and important characteristic of some of the streak diseases. It is a striking symptom of winter blight of tomatoes. This disease has been shown to be due to a combination of two different mosaic diseases, a potato mosaic and a tobacco mosaic. When either of these is transmitted alone to tomato plants chlorosis of the mosaic type results. When transmitted together they cause severe necrosis and produce all the symptoms of the disease which has long been known as winter blight (98).

The third type of symptom is overgrowth. This is less common than chlorosis and necrosis. Overgrowth is associated with certain mosaic diseases such as the mosaic of corn (46). It is one of the symptoms of the winter blight of tomatoes. In the Fiji disease of sugar cane, elongated galls are produced in the phloëm tissues (48). The galls are irregularly distributed in both stalks and leaves. Incipient stages of gall formation are to be found in the leaf and stalk tissues just above and below the terminal bud. The tissues that are to produce overgrowths consist, during the earliest stages of gall formation, of small groups of cells that react differently to stains than do correspondingly healthy tissues. An infectious gall-producing disease recently described by Klebahn probably belongs in this group (44).

Virus diseases do not in most cases cause plants to die. Diseased plants often live as long as healthy ones. In some instances, however, they lower the resistance of plants to other diseases. Sugar-cane mosaic predisposes sugar-cane plants to attack by the red rot fungus (54). Aster yellows lowers the resistance of aster plants to a species of Botrytis that frequently kills yellowed plants (53). Bacteria that are more or less harmless also accompany certain virus diseases (89). It is interesting to note that some of these belong in the group of *Coccaceae* (16).

The symptoms produced by virus diseases are not affected by the manner in which plants are inoculated. The final picture is the same regardless of whether the infective agent is introduced into old or young tissues. Perhaps the most striking characteristic of these diseases is that

they are systemic. They are closely associated with growth and development, producing symptoms at or near growing points. They seldom produce macroscopic symptoms in tissues that are mature when infection takes place. Local symptoms do not appear at the points where plants are inoculated except when the inoculation is made at a growing point. A few exceptional cases must, however, be mentioned. The potato disease known as sprain produces necrosis in the tissues of mature potato tubers, and can be passed by mechanical inoculation from tuber to tuber (8). The ring spot disease of tobacco produces local symptoms in mature leaf tissues at or near points of inoculation (30). Such local infection usually becomes systemic but this is not always the case. In some instances the disease develops only in the tissues immediately surrounding a point of inoculation.

Although systemic most virus diseases of plants produce conspicuous symptoms in leaf and stem tissues only. Little is known of their effects on roots. It has frequently been observed that diseased plants have poorly developed root systems. Whether this is a direct effect of disease or results from a diminution in the food supply that comes from diseased leaves has not been determined in most cases. Allard (1) long ago showed that the roots of mosaic tobacco plants contain the virus of this disease and more recently Goldstein (34) observed that inclusion bodies associated with the disease are present in the cells of roots as well as in those of stems and leaves. Sweet potato mosaic is transmitted through infected roots (76). The fine roots of sandal trees having spike disease die prematurely and their dying has been observed to keep pace with the progress of the disease (22). Sugar-beet roots affected by the curly-top disease are dwarfed and their vascular tissues are discolored and abnormal. On the whole, however, roots are not severely affected by most virus diseases.

One of the most common results of the infection of a plant by a virus disease is stunting. The internodes of stems are usually shortened. In some cases this results in the leaves being brought so close together that rosettes are produced. The maladies of which this symptom is conspicuous are known as rosette diseases. Peach rosette (57), wheat rosette (60), and peanut rosette (5) may be mentioned as examples. In most cases dwarfing of stems probably results from a failure of leaves to elaborate sufficient food. Stems of diseased plants are usually not chlorotic and do not show lesions. Tobacco plants affected with mosaic are stunted but the mottling so characteristic of the disease is confined to leaves. Nevertheless, several of the virus diseases do affect stem tissues

severely. Grass mosaic on some varieties of sugar cane causes lesions throughout stalk tissues (54). The mosaic of corn affects all stalk tissues. The Fiji disease of sugar cane causes galls throughout affected stalks and Klebahn's alloiophylly of *Anemone nemorosa* causes overgrowth in stems (44).

Although few virus diseases are transmitted through seeds they frequently affect fruits. Peaches from yellowed peach trees are insipid and tasteless (87). Cucumber mosaic ruins affected fruits (26) as is shown by the specimen pictured in Fig. 1. Although causing slight damage to fruits tomato mosaic sometimes produces mottling on green fruits (24). Winter blight causes lesions within the green fruits (31).

It is in leaves, however, that virus diseases produce their most conspicuous symptoms. This is perhaps to be expected since chlorophyll, which is seriously affected by these diseases, is produced chiefly in leaves. Virus diseases have been divided into groups on the basis of the symptoms produced in leaves. The mosaics constitute the largest and most important of these groups. All mosaics cause mottling of leaves. Masses of tissue of a light yellowish green color are distributed in the normal green tissues in such a way as to produce a mosaic pattern. The light and deep green colored tissues may be separated from each other by sharp lines of demarkation or the deep green areas may fade more or less gradually into the light green areas. In the common mosaic of tobacco the areas are sharply separated, while in mild mosaic of potato the edges of the light colored spots are diffuse. The chlorotic spots may be small, as in mild mosaic of potato, or they may be very large, as in the mosaic of *Commelina nudiflora* L. They may be circular, as in Aucuba mosaic of potato, or very much elongated, as in grass mosaic on certain varieties of sugar cane. A portion of a mosaic-diseased leaf of the cane variety, Striped Tip, is shown in Fig. 2. The spots may be present when the young leaves of a diseased plant unfold or they may develop in the green tissues after the leaves reach considerable size. It has not been shown in any case why spots develop in particular areas or why all parts of diseased leaves are not equally affected. It is also not known what determines the number or size of the spots on a given leaf. It may be that the deep green tissues adjacent to chlorotic areas acquire a resistance to the disease-producing agent and that the development of such resistance limits the size of spots. No case is known in which plants suffering from a virus disease acquire immunity. Mosaic-diseased perennial plants may live for many years without showing any change in their reaction to the disease. Plants that recover from virus

diseases are not immune to reinfection. Sugar-cane plants that recover from grass mosaic are as liable to reinfection as are plants that have never had the disease. In only one case has a means been found of curing a virus disease. Wilbrink (100) showed that by a hot water treatment of diseased sugar-cane cuttings in which the cuttings were subjected to a temperature of 45–50°C. for 30 minutes and then to a temperature of 50–52 or 53°C. for 30 minutes the virus of sereh disease is destroyed without killing the cuttings.

One of the most interesting characteristics of the chlorotic spots caused by mosaic diseases is that they sometimes show the Liesegang phenomenon. Instead of being uniformly chlorotic throughout, the light green colored parts occur as concentric circles. The circles alternate with normal or almost normal deep green colored tissues. The spots may consist of one or of many chlorotic circles around either a green or a chlorotic center. Those caused by the mosaic disease of peonies frequently show as many as ten chlorotic circles. Those caused by pea mosaic seldom show more than one circle. Elliptical rings are characteristic of the spots of the mosaic disease of *Commelina nudiflora* L. Circular rings such as are shown in Fig. 3 are characteristic for the ring spot disease of tobacco (30). Definite chlorotic circles are not characteristic of the spots of a large number of mosaic diseases but faint or imperfect circles may occasionally be observed in the chlorotic areas caused by many of these diseases.

Baur (11) showed that scions from an immune species of *Abutilon* grafted on a diseased plant of a susceptible species remain healthy but that when healthy scions of a susceptible species are grafted on the healthy appearing scions of the immune species they become diseased. He thus proved that although the scions of immune species never show symptoms of disease the virus of *Abutilon* mosaic nevertheless passes through them. This result is not surprising when it is remembered that certain viruses multiply in plants that never show symptoms of disease and that under certain conditions the symptoms of a number of different virus diseases are masked. Nishamura (65) found that the virus of tobacco mosaic is carried by *Physalis alkekengi* without causing this plant to show symptoms of disease and Elmer (27) obtained a similar result with *Physalis francheti*. Goss and Peltier (38) report that mild mosaic of potato is masked in the new growth produced by plants held at 25°C. while at 15°C. mottling is quite distinct. They found yellow dwarf, on the other hand, to be a high temperature disease. The symptoms of this malady disappeared in growth produced at 15°C. but were

severe in growth produced at 25°C. Bennett (15) showed that in certain varieties the symptoms of red raspberry mosaic are masked at 28°C. But the symptoms of the raspberry curl disease remain characteristic at all temperatures which permit the growth of affected plants.

Number of Virus Diseases

No one knows the exact number of different mosaic diseases that are prevalent even on our most important cultivated crops. It is usually assumed that each of the mosaics observed on plants not closely related is distinct. This assumption has, however, been brought into question by the work of Elmer (27), who succeeded in obtaining mosaic transmission among species belonging to 15 different families of plants. For example, he infected tobacco plants with virus obtained from sugar-cane, bean, raspberry, cucumber, Zinnia, Calendula, celery, milkweed, columbine, spurge, *Martynia*, *Abutilon* and a few other plants. He did not prove, however, that the diseases transmitted from each of these plants are the same. The fact that a given mosaic disease can be carried to a number of different plants is not proof that the mosaics commonly present on these plants are identical. Most of Elmer's successful interfamily transfers are probably to be explained by the fact that mosaic diseases have wide and overlapping host ranges. It must nevertheless be admitted that at the present time we are not quite sure that the different mosaic diseases may not be strains of one disease modified by transmission through different host plants. Elmer's success in transmitting mosaic diseases is suggestive of possibilities in this direction. Walker (99), working with the mosaics of cucumber, tomato, and the cultivated ground cherry, has shown that the properties of a virus from a given plant may be decidedly changed by transferring it to another host. The infective agents in juice from diseased tomato and ground cherry plants in tests on aging, drying, heating, dilution, and treatment with alcohol reacted in the same manner. The active agent in juice from mosaic cucumber, on the other hand, is incapable of withstanding aging, drying, heating, and treatment with alcohol to a degree comparable to that of the agents from the other two plants. But the infective principle in juice from ground cherry plants previously inoculated with the virus from mosaic cucumber plants gave the same reactions as the active principle in juice from ground cherry plants inoculated with virus from mosaic ground cherries. And, in like manner, the properties of the virus from cucumber plants were found to be the same regardless of whether the plants were inoculated from mosaic cucumbers or from

mosaic ground cherries. Another excellent example of the modification of a virus by passage through particular host plants is that reported by Carsner (20) for the virus of the curly-top disease of sugar beets when passed through either of the three species *Chenopodium murale, Rumex crispus* or *Suaeda moquini.* If carried from these plants back to sugar beets the virus causes a mild or attenuated form of the curly-top disease. The attenuated strain of curly-top occurs naturally in sugar-beet fields.

Regardless of what the relationship between mosaic diseases on different host plants may be, a considerable number of them show constant differences when inoculated into plants of the same species and variety. Schultz and Folsom (80) have shown the existence of at least five different symptomatically distinct mosaic diseases of potato. Johnson (42) recognizes eight different mosaic diseases that are transmissible to tobacco, and Bennett (15) has recently shown that three different mosaics attack raspberries. At least two different mosaic diseases occur on corn (51). Fernow (29) in interspecific transmission experiments on nineteen different species of plants shows the existence of eight different mosaic diseases. He recognizes these diseases by their different host ranges and by the different symptoms they produce when inoculated into the same species.

Mosaic diseases have been transmitted to or have been observed on a large number of plants. An incomplete list of susceptible species is given below.

Gramineae	
Zea mays L.	corn
Tripsacum laxum Wash.	Guatemala grass
Syntherisma sanquinalis	
Syntherisma pruriens (Trin.) Arthur	crab grass
Saccharum officinarum L.	sugar cane
Holcus sorghum L.	sorghum
Saccharum navenga	wild sugar cane
Pennisetum glaucum R. Br.	pearl millet
Paspalum boscianum Flügge	bull grass
Panicum miliaceum L.	millet
Panicum dichotomiflorum Michx.	
Oryza sativa L.	rice
Miscanthus sinensis Anderss.	
Eleucine indica (L.) Gaertn.	goose grass
Echinochloa crusgalli L.	barnyard grass
Echinochloa colona (L.) Link	
Chaetochloa magna	giant foxtail grass
Chaetochloa verticillata L.	foxtail grass

Chaetochloa lutescens	
Andropogon sorghum virgatus (Hack.) Piper	Tunis grass
Andropogon sorghum sudanensis Piper	Sudan grass
Andropogon sp.	wonder forage grass
Commelinaceae	
Commelina nudiflora L.	hono hono grass
Liliaceae	
Lilium longiflorum Thumb.	
Dianella odorata Bl.	
Hyacinthus sp.	
Tulipa sp.	
Amaryllidaceae	
Eucharis amozonica Lindl.	
Hippeastrum equestre Herb.	
Hippeastrum johnsonii Bury.	
Narcissus sp.	
Musaceae	
Musa cavendishii Past.	Chinese banana
Cannaceae	
Canna indica L.	
Uticaceae	
Humulus lupulus L.	hops
Polygonaceae	
Rumex obtusifolius L.	Rumex
Rumex britannica L.	water dock
Fagopyrum esculentum Moench.	buckwheat
Chenopodiaceae	
Spinacia oleracea L.	spinach
Beta vulgaris L.	beet
Amaranthaceae	
Amaranthus retroflexus L.	pigweed
Phytolaccaceae	
Phytolacca decandra L.	pokeweed
Caryophyllaceae	
Dianthus barbartus L.	sweet william
Ranunculaceae	
Paeonia albiflora Pallas.	peony
Aquilegia caerulea James	columbine
Aquilegia canadensis L.	columbine
Lauraceae	
Persera gratissima Gaertn.	avocado
Cruciferae	
Raphanus sativus var. *caudatus*	rat-tailed radish
Brassica rapa L.	turnip
Brassica pekinensis Rupr.	Chinese cabbage
Brassica japonica Hort.	mustard
Brassica campestris L.	

Leguminoseae	
Vigna catjang Walp.	catjang
Vigna sinensis Endl.	cowpea
Vicia faba L.	broad bean
Trifolium repens L.	white clover
Trifolium pratense L.	red clover
Trifolium incarnatum L.	crimson clover
Trifolium hybridum L.	alsike clover
Soja max Piper	soy bean
Pisum sativum L.	garden pea
Phaseolus vulgaris L.	bean
Phaseolus radiatus var. *aurea*	Azuki bean
Phaseolus lunatus L. *macrocarpus*	
Phaseolus limensis Macf.	Lima bean
Phaseolus acutifolius Gray	Tepary bean
Melilotus officinalis Lam.	yellow sweet clover
Melilotus alba Desr.	white sweet clover
Medicago sativa L.	alfalfa
Medicago lupulina L.	hop clover
Medicago arabica Huds.	spotted bur clover
Lathyrus odoratus L.	sweet pea
Laburnum vulgare (*chrysophyllum*)	
Arachis hypogoea L.	peanut
Euphorbiaceae	
Euphorbia preslii Guss.	spurge
Anacardiaceae	
Rhus typhina L.	sumac
Celastraceae	
Evonymus japonica L.	spindle tree
Vitaceae	
Vitis labrusca L.	grape
Malvaceae	
Abutilon theophrasti Medic.	abutilon
Abutilon thompsoni	abutilon
Hibiscus esculentus L.	okra
Passifloraceae	
Passiflora sp.	passion flower
Umbelliferae	
Apium graveolens L.	celery
Oleaceae	
Fraxinus pubescens aucubifolia	ash
Ligustrum vulgare L.	privet
Asclepiadaceae	
Asclepias syriaca L.	milkweed
Convolvulaceae	
Ipomoea batatas Poir.	sweet potato
Labiatae	
Nepeta cataria L.	catnip

Solanaceae	
Capsicum annuum L.	pepper
Datura fastuosa L.	
Datura meteloides Dunal.	
Datura quercifolia	
Datura stramonium L.	Jimson weed
Datura tatula L.	
Hyoscyamus niger L.	henbane
Lycopersicon esculentum Mill.	tomato
Lycopersicon pimpimellifolium Mill.	currant tomato
Nicandra physaloides Gaertn.	apple of Peru
Nicotiana alata Link and Otto	
Nicotiana forgetiana Sander	
Nicotiana glauca Graham	
Nicotiana glutinosa L.	
Nicotiana langsdorfi Weinm.	
Nicotiana longiflora Cav.	
Nicotiana plumbaginifolia	
Nicotiana paniculata L.	
Nicotiana rustica L.	
Nicotiana sylvestris Spegaz. and Comes	
Nicotiana tabacum L.	tobacco
Petunia violaceae Lindl.	petunia
Physalis angulata L.	
Physalis alkekengi L.	winter cherry
Physalis francheti Hort.	
Physalis heterophylla Nees.	
Physalis longifolia Nutt.	
Physalis pubescens L.	
Physalis minima L.	
Physalis subglabrata Mackenzie and Bush	
Physalis virginiana Mill.	
Solanum aculeatissimum Jacq.	apple of Sodom
Solanum atropurpureum	
Solanum carolinense L.	
Solanum dulcamara L.	
Solanum ferox L.	
Solanum integrifolium	
Solanum nigrum L.	
Solanum tarvum L.	
Solanum melongena L.	eggplant
Solanum verbascifolium	
Solanum tuberosum L.	potato
Martyniaceae	
Martynia louisiana Mill.	unicorn plant
Cucurbitaceae	
Benincasa hispida Cog.	white gourd
Citrullus vulgaris Schrad.	watermelon

Cucumis sativus L.	cucumber
Cucumis anguria L.	West Indian gherkin
Cucumis grossulariaeformis Hort.	
Cucumis melo L.	
Cucumis metulliferus Mey.	
Cucurbita maxima Duchesne	
Cucurbita moschata Duchesne	crook neck squash
Cucurbita pepo L.	sugar pie pumpkin
Momordica elaterinum A. Rich.	squirting cucumber
Momordica involucrata E. Meyer	
Momordica charantia L.	
Momordica balsamina L.	
Lagenaria vulgaris Ser.	
Lagenaria leucantha Rusby	
Luffa acutangula Roxb.	
Luffa cylindrica Roem.	
Melothria scabra Naudin	
Micrampelis lobata (Michx.) Greene	wild cucumber
Sicyos angulatus L.	
Trichosanthes anguina L.	
Lobeliaceae	
Lobelia erinus var. *gracilis*	Lobelia
Compositae	
Ambrosia trifida L.	great ragweed
Calendula officinalis L.	marigold
Cineraria sp.	
Heliopsis scabra Dunal.	
Helianthus debilis Nutt.	
Hypochaeris radicata L.	
Stokesia laevis Hill	Stokes aster
Zinnia elegans Jacq.	
Lactuca sativa L.	lettuce

A list of some virus diseases outside of the mosaic disease group is given below.

Yellows of peach (87)
Yellows of China asters (53)
Yellows of strawberry (69)
Witches' broom of strawberry (102)
Rosette of wheat (60)
Rosette of peanuts (95)
Rosette of peach (57)
Little peach of peach (87)
Leafroll of potato (80)
Spindle tuber of potato (80)
Sprain of potato (8)
Witches' broom of potato (39)
Streak of potato (7)
Streak of raspberry (15)
Curl of raspberry (15)
Fiji disease of sugar cane (56)
Sereh disease of sugar cane (56)
Curly-top of beets (9)
False blossom of cranberry (84)
Spike of sandal (22)
Bunchy-top of bananas (32)
Crazy-top of cotton (23)
Spotted wilt of tomatoes (85)
Nettle-head of hops (77)

Transmission

Such diseases as tobacco mosaic and cucumber mosaic are transmitted with great readiness and the agents that cause them are filterable. Unbelievably small quantities of juice from a mosaic tobacco plant are sufficient to transmit the disease when rubbed into a wound of a healthy plant. Beijerinck (14) who first showed that tobacco mosaic is caused by a filterable virus states that the quantity of filtered juice necessary to infect plants is extremely small. Allard (2) readily obtained infection with juice diluted to 1 part in 10,000 parts of water. The transfer of such small quantities of juice as are carried in the most careful handling of plants frequently spreads the disease. Tobacco juice allowed to ferment and kept at room temperature as long as 1 year is still highly infectious. In dried tobacco leaves the causal agent remains infective for many years. But in spite of the extreme infectiousness of this virus it enters healthy plants only through wounds. When sprayed on unwounded leaves it does not cause disease. The smallest wound that can be made by the prick of a fine needle or by the breaking off of a few trichomes is sufficient to permit its entry. Some virus diseases must be classed among the most infectious maladies to which plants are susceptible but the group as a whole is not highly infectious. Sugarcane mosaic (18) and the curly-top (83) of beets are transmitted by rubbing juice from diseased plants into wounds made in healthy plants. But in most transmission experiments with these viruses only a very small percentage of the plants inoculated became diseased. Several virus diseases of the potato are not readily transmitted by mechanical means. Leafroll (79) and witches' broom (101) have not been transmitted mechanically except by grafting, and grafting is the only method by which Abutilon mosaic (10), aster yellows (53), peach rosette (87), peach yellows (87), spike of sandal (22) and a number of other virus diseases of plants have been mechanically transmitted. Baur (10) concluded that Abutilon mosaic must be caused by a non-living entity because he was unable to transmit it in any way except by grafting. He could not understand how it would spread to healthy plants in nature. From what we now know it seems probable that some insect may be capable of spreading this disease.

Although the virus of tobacco mosaic is present in roots, stems, leaves, and flowers it is not transmitted through seeds and this is true of most other virus diseases of plants that have been carefully studied. Each new crop produced from seed is, therefore, free from disease. This is

fortunate, for it makes control much easier than it would be, if they were seed-borne. There are, however, some virus diseases that are regularly transmitted through seeds. This is true of lettuce mosaic (41) and of the mosaics that attack a number of different leguminous plants. The common bean, the soy bean, the Lima bean, the garden pea, and several different clovers have been shown to transmit mosaic through their seeds (24). Most virus diseases seriously reduce the yield of seeds and some of them cause sterility. Peach pits produced on branches having the yellows disease will not germinate (87). Aster yellows (52) causes sterility in a large number of plant species. In no case has proof been brought that any virus disease spreads by growth through the soil in which plants are grown or by the splashing of rain from diseased to healthy plants. In this respect virus diseases differ from bacterial diseases of plants.

The most important factor in the spread of virus diseases is their transmission by insects. Many have been proved to be so transmitted and it is probable that all are spread in this way. Sucking insects are the most common vectors. If there were no insects, the virus diseases of plants would be of little consequence. The more these maladies are studied, the more evident it becomes that epiphytotics of virus diseases are related to the fortunes of insect colonies of the species concerned in the transmission of these diseases. In some cases the spread of virus diseases by insects is doubtless nothing more than a simple mechanical transfer. Some diseases are spread by several different insect species. Mild mosaic of potato is transmitted by the potato aphid (*Macrosiphum solanifolii* Ash.) (80), the peach aphid (*Myzus persicae* Sulz.) (81), and the buckthorn aphid (*Aphis abbreviata* Patch) (78); cucumber mosaic by the melon aphid (*Aphis gossypii* Glover) (26), the spotted cucumber beetle (*Diabrotica duodecimpunctata* Oliv.), the striped cucumber beetle (*Diabrotica vittata* Fabr.) and two species of mealy bugs (*Pseudococcus maritimus* Ehr., *Dactylopius sp.*). Spinach mosaic (58) is transmitted by the tarnished plant bug (*Lygus pratensis* L.), the bean aphid (*Aphis rumicis* L.) and the potato aphid. Tobacco mosaic is reported to be spread by the tobacco aphid (*Macrosiphum tabaci* Pergande) (1), the peach aphid (4), the tobacco horn worm (*Protoparce sexta* Johanssen) (27) and a mealy bug (*Pseudococcus citri* Risso) (68). Raspberry mosaic is spread by two aphids (*Aphis rubiphila* Patch and *Amphorophora rubi* Kalt.) (72). Potato leafroll is reported to be transmitted by the potato aphid (79), the peach aphid, the elm leafhopper (*Typhlocyba ulmi* L.) (62) and the tarnished plant bug (28).

A number of virus diseases have been shown to be spread by only one insect but further studies may prove that other insects transmit some of them. *Aphis leguminosae* Theobald transmits the rosette disease of peanuts (95). Clover mosaic is transmitted by (*Macrosiphum pisi* Kalt.) (25), bean mosaic by the potato aphid (64), lettuce mosaic by the peach aphid (41), cowpea mosaic by the bean-leaf beetle (*Ceratoma trifurcata* Forst.) (86) and the bunchy-top disease of bananas by the aphid, *Pentalonia nigronervosa* Coq. (32 and 66).

The peach aphid is perhaps the best known example of an insect that is capable of transmitting several different virus diseases. This aphid transmits spinach mosaic, tobacco mosaic, lettuce mosaic, sugar-beet mosaic, and potato mosaic. In all of these examples of insect transmission there is little evidence that the insects act in any way other than to make a simple mechanical transfer of the diseases.

In other cases, however, a specific relationship seems to exist between certain insects and the diseases which they carry. Curly-top of sugar beets is transmitted by the beet leafhopper, *Eutettix tenellus* Baker, but is not transmitted by other beet insects (9). Sugar-beet mosaic, on the other hand, is transmitted by the peach aphid but not by the beet leafhopper (75). The leafhopper, *Balclutha mbila* Naude, spreads the South African streak disease of corn (94), which is not carried by the corn leafhopper, *Peregrinus maidis* Ashm., or by the corn aphid, *Aphis maidis* Fitch. Each of these insects do, however, carry other virus diseases to corn. *Peregrinus maidis* transmits corn mosaic to corn (50) while *Aphis maidis* transmits the grass mosaic (sometimes designated as sugar-cane mosaic) to corn (17). *Peregrinus maidis* is unable to transmit grass mosaic to corn (51). Each of these three corn insects transmits a different virus disease. The three diseases seem to be specific for the insects that carry them. Rice mosaic is spread by the leafhopper, *Nephotettix apicalis* Motsch. (96). Several other species of leafhoppers that feed on rice are not able to transmit this disease. Although unable to live for more than a few days on most commercial varieties of sugar cane *Aphis maidis* readily transmits grass mosaic to these varieties. *Aphis sacchari* Zehntn. which thrives on sugar cane is unable to transmit the disease (54). The aster yellows disease is transmitted by the leafhopper, *Cicadula sexnotata* Fall. (49), but is not transmitted by several other aster leafhoppers or by the peach aphid which transmits so many different mosaic diseases (53). These facts suggest that an intimate, specific, biological relationship exists between certain virus diseases and their insect vectors. There is other experimental evidence that supports this

view. Four cases are known in which insects that carry virus diseases are incapable of transmitting these diseases immediately after first feeding on diseased plants. A definite period of time must elapse before they can transmit the viruses they carry. The diseases referred to are curly-top of beets, streak of corn, leafroll of potato, and yellows of asters. Smith and Boncquet (89) in 1915 reported that after feeding on a diseased plant for the first time, 24 to 48 hours must elapse before *Eutettix tenellus* can transmit curly-top to a healthy beet plant. This finding was confirmed by Severin (82) and others. Severin showed that the length of the incubation period varies with the temperature at which the insects are kept. High temperatures shorten the period. For insects kept at 100°F. the incubation period was found to be about 4 hours. An incubation period of similar length was found by Storey (93) for the virus of the streak disease of corn in the leafhopper, *Balclutha mbila*, and by Elze (28) for the virus of leafroll of potato in the aphid, *Myzus persicae*. A much longer incubation period was obtained by Kunkel (53) for the virus of aster yellows in the leafhopper, *Cicadula sexnotata*. The minimum incubation period found in this case was 10 days. Fig. 4 shows 27 aster plants used in an experiment for determining the length of the incubation period of the aster yellows virus in the aster leafhopper. One hundred adult virus-free leafhoppers were allowed to feed for 1 day on the small diseased plant shown in the upper left corner of the figure. They were then confined for 1 day on each of 26 healthy young aster plants. The plants shown in the picture are arranged in the order in which they were exposed. The photograph was taken some weeks after the last plant was exposed and shows that the 11 plants on which the insect colony fed during the 11 days following their confinement on the diseased plant remained healthy. The other plants on which the insects fed after this time became diseased as is shown by the upright habit of growth of their leaves and by stunting.

The length of time some of the virus diseases have been shown to be carried by insects is further evidence of a specific relationship. Stahl and Carsner (90) showed that virus-bearing individuals of *Eutettix tenellus* retain their ability to infect for many days after they have been removed from susceptible plants. Storey found that the virus of the streak disease of corn is retained by *Balclutha mbila* for a very long period of time. One individual retained the virus for 4 months during which time the insect fed on healthy sugar cane. The virus of leafroll of potato was retained by the peach aphid for a period of 1 week. The virus of aster yellows was shown to be retained by *Cicadula sexnotata*

for a period of 2 months during which time the insect was confined on rye plants that are immune to the disease. Certain individuals were, however, shown to lose their ability to infect healthy plants after a few days. Some individuals of *Eutettix tenellus* are incapable of carrying the curly-top virus and likewise some individuals of *Balclutha mbila* are unable to spread the streak disease of corn. All of 28 individuals of *Cicadula sexnotata* that were carefully tested were found capable of spreading aster yellows.

We do not yet know what determines the specific relationship that has been shown to exist between certain virus diseases and their insect carriers. But although none of the insect carriers of these diseases have been proved to suffer any ill effects from the diseases they transmit, it is nevertheless possible, if not even probable, that the insects are hosts of the disease-producing entities and that the viruses multiply in their insect carriers. All of the insects shown to be specific carriers of virus diseases belong in the group of the Homoptera. But the specificity of these insects has not been shown to be related in any way to the manner in which they feed.

Virus diseases that are highly infectious are doubtless transmitted mechanically by almost any insect that visits and feeds upon susceptible plants but even in these cases certain insect carriers may be specific. In this connection it is interesting to recall that although spinach blight is transmitted by several different insects, one of its carriers, the aphid, *Macrosiphum solanifolii*, seems to be specific for the disease. McClintock and Smith (58) showed that the spinach blight virus is retained by the aphid when kept for some time on non-susceptible plants and that the disease is inherited up to the fourth generation by individuals parthenogenetically produced. In no case has a plant virus disease been shown to be transmitted through the eggs of an insect carrier and there is no evidence that viruses are spread from one insect to another by contact. They are carried from year to year by plants or plant parts kept in greenhouses or other protected places, by perennial plants, and by insect vectors that live over winter.

The spread and severity of most virus diseases of plants vary with fluctuations in the number of insects of the species that transmit them. Weather conditions, insect parasites, and abundance of food plants are factors of great importance in determining the prevalence of insects and the spread of virus diseases. The geographical distribution of insect species determines the geographical range of many virus diseases, and the likes and dislikes of insect species frequently determine the host

ranges of these maladies. Virus diseases are so dependent on insects for their spread that epiphytotics of these maladies can be understood only when the life histories, habits, host plants, rates of increase, and physiology of the insects that spread them are known.

Etiology

A number of different organisms have been described as associated with or as causing one or more virus diseases of plants. Such claims, in all cases, either have been disproved or remain unconfirmed and need not be mentioned here. Olitsky (67) thinks he obtained evidence of multiplication *in vitro* of the active agent of mosaic disease of tobacco and tomato. Other workers (36, 61, and 70) have been unable to confirm his conclusions and up to the present time no plant virus has been proved to multiply except when associated with living plant cells. Johnson (43) believes he was able to start a new virus disease of tobacco with juice from healthy potato plants but it has not been proved that the potatoes he used were not masked carriers of the virus he obtained.

Many attempts have been made to discover a causal organism in the tissues of plants affected by virus diseases. In early studies on tobacco mosaic, an effort was made to find a bacterium that would produce the disease. The search for schizomycetes that will give the solution of the whole virus disease problem has not yet ended. Iwanowski (40) was the first to make a careful study of diseased tobacco tissues. In his search for a bacterium he discovered that certain of the cells in diseased leaves contain plastic ameboid bodies such as are shown in Fig. 5. The bodies are never found in the cells of healthy plants. He noted their striking resemblance to amebae and states that he was led to wonder whether they might not be of etiological significance. Since, however, the entity that causes tobacco mosaic can pass through the pores of a porcelain filter he concluded that the ameboid bodies must be a result rather than the cause of the disease. In the same year that Iwanowski published his paper describing the ameboid bodies of tobacco mosaic Negri (63) reported the presence of small bodies in the brain cells of animals suffering from rabies. Little attention was given by other workers to the bodies described by Iwanowski. They apparently were not observed by other students during some 20 years following the publication of his paper.

In 1910 Lyon (55) described a disease of sugar cane which was then prevalent in Fiji and to which he gave the name Fiji disease. So far as the writer is aware this malady is unlike any other known plant disease

but there is little doubt that it belongs in the virus group. It is an infectious systemic gall disease. Elongated galls such as are pictured in Fig. 6 are found at irregular intervals in the phloëm tissues of leaves and stalks. Lyon found small spherical and irregular shaped bodies in the gall cells. These he believed to represent a parasitic organism but found that they differed from any known intracellular parasites. These bodies were given no more attention by the students of virus diseases than were the bodies reported by Iwanowski. In 1921 Kunkel (46) described and pictured intracellular bodies that are associated with the mosaic disease of corn. The bodies were found in the cells of the chlorotic tissues of diseased corn plants but were never observed in the cells of the normal green tissues of such plants or in the cells of healthy plants. Corn cells are larger than those of tobacco or sugar cane and are, therefore, more favorable for cytological study. The succulent stalk tissues were found to be especially suitable for observations on the bodies associated with this disease and they were studied in both fixed and living cells. Successive stages in the development of the disease were observed in affected tissues. Chlorosis is the first symptom of this disease in green tissues. In non-green stalk tissues the first symptom is a slight discoloration. Small ameboid bodies are always present in the cells of such tissues. As the bodies increase in size the cells that contain them enlarge and finally collapse and die. It was observed that protoplasmic streaming is more rapid in diseased than in healthy cells. The bodies are usually very closely associated with the corn cell nucleus as is shown in Fig. 7. In 1922 Kunkel (45) reported that similar bodies are associated with the mosaic disease of *Hippeastrum equestre* Herb. In this disease also the bodies are associated with the cell nuclei as shown in Fig. 8. The next year McKinney, Eckerson and Webb (59) described intracellular bodies that are associated with a mosaic of *Hippeastrum johnsonii*. The same authors found and described inclusion bodies associated with wheat rosette (60). These bodies, two of which are shown in Fig. 9, bear a striking resemblance to the bodies of the Fiji disease of sugar cane. The following year Kunkel published the results of a cytological study of the mosaic disease of *Hippeastrum* (47) and the Fiji disease of sugar cane (48). The bodies of *Hippeastrum* mosaic are smaller than those of corn mosaic. In both diseases they are less closely associated with the plant nuclei than are the bodies of corn mosaic. Studies on the intracellular bodies of tobacco mosaic were published by Goldstein (33) in 1924 and by Rawlins and Johnson (73) in 1925. The former author in work on living cells observed that the

bodies frequently change both their form and position. Such changes suggest that they may represent a living organism or perhaps an aggregate of living organisms. Goldstein (34) has recently made a thorough-going cytological study of the leaves and growing points of mosaic-diseased tobacco plants. She concludes that a distribution of the intracellular bodies, or x-bodies as she designates them, is accomplished by the division of tobacco cells. She states that "each new daughter cell formed contains before it is separated from its sister cell one or more x-bodies." She also brings evidence that x-bodies divide by constriction. These conclusions are in agreement with those reached by Kunkel (48) in studies on the bodies of the Fiji disease. The bodies associated with this disease were observed in different stages of division. Late stages in what appears to be division by constriction are common in young galls. Further evidence of division is brought by the large number of cells that contain two bodies. The nuclei of such cells were observed in mitotic division and large numbers of young cells were found to contain one body each. Stages in the processes by which the bodies are believed to be distributed are shown in Fig. 10. Purdy (71) found that the virus of tobacco mosaic multiplies in leaves inoculated after they are detached from the plants that bear them. She also observed that intracellular bodies are produced in the cells of such detached leaves. Convincing evidence that x-bodies divide and are distributed by successive host cell divisions was obtained by Goldstein (35) in her studies on the mosaic disease of Dahlias. She observed and photographed numerous stages of what appear to be division of the bodies by constriction. She also pictures x-body-containing cells in mitotic division. Her conclusion that the bodies are distributed by cell division seems justified by her observations. The evidence is good that the bodies of Fiji disease, tobacco mosaic and Dahlia mosaic divide and that they are distributed by cell division. This behavior suggests that they are not waste products or inert bodies resulting from a diseased condition in the cells that contain them. The demonstration that inclusion bodies divide brings the best evidence which we now possess that they may represent stages in the life cycles of living organisms. But final proof that they are living has not yet been given.

It has sometimes been urged that the intracellular bodies can hardly be of etiological significance since they are much too large to pass through the pores of a porcelain filter. It must be remembered, however, that the units of which they are composed may be very minute and that the full-grown inclusion bodies may represent very large

colonies of those units. The tiny swarm spores of certain myxomycetes are far too small to be observed with the naked eye. But the full grown plasmodia which some of them produce may cover as much as a square foot of surface and are many millions of times larger than the units that initiate them.

The manner in which intracellular bodies are first produced in plant cells at or near growing points has not yet been determined. The causative agents of certain virus diseases are known to travel through plant tissues with great rapidity. This suggests that they may move through the vascular tissues. Severin (83) showed that the virus of the curly-top of beets will move through as much as 7 inches of beet leaf petiole in ½ hour. Storey (93) found that the virus of the streak disease of corn moves rapidly through leaf tissue. The highest rate he observed in movement down a leaf was 40 centimeters in 3 hours. Beijerinck long ago suggested that the virus of tobacco mosaic may be distributed through the phloëm tissues. Several observations seem to support this view. In tobacco mosaic, curly-top of beets, yellows of asters and a number of other virus diseases the first symptom is a clearing of veins in young leaves. This seems to be due to a stimulation of growth in cells of the phloëm. The galls of the Fiji disease are always produced in phloëm tissues. Phloëm necrosis is one of the most constant symptoms of the leafroll disease of potato. While the agents causing virus diseases doubtless travel through vascular tissues, there is evidence that some of them can pass through other tissues as well. It is an established fact that tobacco mosaic can be transmitted without injecting the virus into vascular elements. Infection occurs readily through trichomes. There are probably two stages in the distribution in plant tissues of the agents causing virus diseases. The initial distribution is rapid and takes place soon after inoculation. It is probably accomplished by passage through vascular tissues of entities beyond the range of microscopic vision. It results in the infection of cells at or near growing points. The second stage may be accomplished, in some diseases at least, through the division of intracellular bodies and their distribution by cell division.

Intracellular bodies are now known to be associated with many plant maladies belonging to the mosaic type of disease. Smith (88) has recently shown that bodies similar to those of tobacco mosaic are present in cells in the chlorotic areas of mosaic-diseased plants of *Petunia*, *Phytolacca decandra* L., and *Evonymus japonica* L. and that bodies similar to those of corn mosaic accompany the mosaic of *Datura stramonium* L. In all of these diseases the intracellular bodies bear a striking resem-

blance to amebae. They are plastic, protoplasm-like bodies, and although they have not yet been demonstrated to possess nuclei, they sometimes contain deeply staining granules. They are usually vacuolate. They show characteristic differences in the different diseases with which they are associated but are enough alike to indicate that they are closely related structures. In some instances they are associated with host cell nuclei and bear a striking resemblance to some well-known intracellular parasites of plants. Students of the mosaic diseases know that intracellular bodies similar to those that have been described are associated with many diseases for which they have not yet been reported. Further studies will doubtless add much to our knowledge of these peculiar structures and to an understanding of their significance for the diseases with which they are associated. But whatever may be their significance it is evident that they are of considerable diagnostic value. Similar inclusions have not been found in association with any disease caused by a known organism and they are not associated with the noninfectious type of chlorosis in plants (88). They are doubtless related to the somewhat similar inclusion bodies associated with certain virus diseases of animals.

SUMMARY

In summarizing, it may be stated that the virus diseases of plants are infectious, systemic, chronic diseases causing chlorosis, necrosis, and overgrowth. They produce symptoms in tissues of a rather definite age. In most instances they develop in young tissues. They seldom kill plants though they may lower their resistance to other diseases that do kill them. They do not produce active immunity. Intracellular bodies are associated with many of these diseases. A very few of them are seed-borne. Some virus diseases are highly infectious and have been shown to be caused by filter-passing agents. Others are not highly infectious and have not been transmitted except by grafting or by insects. The fact that they are insect-borne diseases should be emphasized. In a considerable number of cases a specific relationship exists between the diseases and their insect carriers. The virus diseases constitute a distinct group of plant maladies which have characteristics that separate them from other plant diseases including those caused by bacteria. These characteristics give as much support to the view that the virus diseases of plants are not due to bacteria as does the fact that many of them are caused by agents that are filterable. They also indicate that this fifth group of infectious plant diseases is not closely related to any of the diseases caused by known organisms.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. A cucumber affected by the cucumber mosaic disease. (After Doolittle.)

Fig. 2. Mosaic disease on a portion of a sugar-cane leaf. (After Kunkel.)

Fig. 3. Ring spot disease on a tobacco leaf.

Plate 2

Fig. 4. Aster plants used in demonstrating the existence of an incubation period for the virus of aster yellows in the aster leafhopper. The same colony of leafhoppers was confined on each plant for 1 day and the plants were exposed in the order shown in the picture. All plants were healthy except the first which had the aster yellows disease. The leafhoppers were virus-free until they fed on this plant. The picture was taken some weeks after the experiment was ended and shows that the 11 plants on which the colony was confined during the 11 days following their confinement on the first plant remained healthy while the other plants became diseased. (After Kunkel.)

Fig. 5. The intracellular bodies associated with tobacco mosaic (After Iwanowski.)

Fig. 6. Galls of the Fiji disease of sugar cane. (After Lyon.)

Plate 3

Fig. 7. An intracellular body of corn mosaic attached to a corn nucleus. (After Kunkel.)

Fig. 8. Inclusion bodies of *Hippeastrum* mosaic in cells of leaf tissue. (After Kunkel.)

Fig. 9. The inclusion bodies associated with wheat rosette. (After McKinney, Eckerson and Webb.)

Fig. 10. Stages in the distribution of the bodies of Fiji disease by cell division. (After Kunkel.)

PLATE 1

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PLATE 2

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PLATE 3

VIRUS DISEASES OF BACTERIA—BACTERIOPHAGY

JACQUES J. BRONFENBRENNER

CHAPTER X

VIRUS DISEASES OF BACTERIA—BACTERIOPHAGY

INTRODUCTION

The inclusion of bacteriophagy or transmissible lysis of bacteria, frequently called the Twort-d'Herelle phenomenon, in a discussion of filterable virus diseases may be opposed by certain workers. The fact, however, that d'Herelle believes the phenomenon to be a disease of bacteria produced by an autonomous, ultramicroscopic, corpuscular virus is sufficient reason for including a discussion of it in a book on filterable viruses. Many of the author's ideas are not in agreement with those of d'Herelle. Nevertheless, at the present time ample justification exists for considering bacteriophagy, particularly in view of the general remarks concerning filterable viruses in Chapter I.

Historical Review

While engaged in attempts to cultivate filterable viruses upon artificial media, Twort made a chance observation that the colonies of micrococci growing on agar as a contamination present in the glycerolated vaccine virus often tended to become "glassy" and transparent (208). Such colonies could not be subcultured as a streak, but if they were plated out, they would occasionally give rise to visible colonies which, in turn, on prolonged incubation showed the tendency to become glassy. When pure cultures of micrococci were contaminated with minute amounts of this glassy material, they also became glassy. This process could be carried on indefinitely, suggesting that the glassy material multiplied, and that this transformation was "not unlike an acute infectious disease of micrococci." Twort thought that the active agent causing this disease might be some kind of a filterable virus, since the filtrates of glassy material (diluted 1:1,000,000 with water or saline solution) were just as active as was the original substance. He thought that this virus probably belongs "somewhere in the vast field of life more lowly organized than the bacterium or amoeba," and that "it may be living protoplasm that forms no definite individuals, or an enzyme with power of growth." However, since in many instances this transformation took

place also in cultures which for months continued growing normally, Twort believed that there was also a possibility that the glassy material arose spontaneously from normal micrococci.[1] According to this view, such endogenously arising glassy material might be "either a stage in the life history [of micrococcus] which will not grow on ordinary media, but stimulates fresh cultures of micrococcus to pass into the same stage, or an enzyme secreted by the micrococcus which leads to its own destruction and the production of more enzyme." "Similar though not such definite results were obtained with the micrococcus and a member of the colon-typhoid group of bacilli which were obtained from the intestinal mucous membranes of a dog suffering from acute distemper, and there is some evidence that the difficulty often experienced in isolating certain unknown, pathogenic microörganisms may be due to the same cause" (208). Two years later, d'Herelle made similar observations while attempting to discover the cause of the diminution in the rate of growth of *B. dysenteriae* in the intestinal contents of convalescents (110). He found that minute quantities of dejecta (or of the fecal filtrates) of convalescents inhibited *in vitro* the growth of *B. dysenteriae*, while similar material collected in the early stages of the disease gave rise to profuse growth of cultures. Since the inhibiting agent did not diminish in its activity through dilution effected by passage from one culture of *B. dysenteriae* to the next for hundreds of generations and since through these transfers the agent, on the contrary, often became more active through adaptation, d'Herelle concluded that he was dealing with a living, multiplying lytic agent. Following the progress of lysis under a microscope, he observed that this agent is an organized, corpuscular parasite (protobe) which is attracted to bacteria by virtue of chemotaxis. "The protobe, then, penetrates the peripheral zone of the bacterium (the openings made remaining open) and multiplies within the bacterial protoplasm, forming a colony composed of a number of elements varying with the conditions of the moment. When process of multiplication is completed, the parasitized bacterium ruptures and undergoes a sudden dissolution. This liberates the young protobes and each of the latter is then ready to repeat the process and parasitize the nearest bacterium. Thus the cycle begins anew, and continues until all the bacteria present in the medium have disappeared" (123—p. 370). D'Herelle has named this ultramicroscopic parasite "Bacteriophagum

[1] This phenomenon. he thought, was analogous to cancer formation in higher cells.

intestinale" or "Protobios bacteriophagus."[2] The parentheses in this quotation are d'Herelle's.

Distribution in Nature

Since the appearance in 1917 of d'Herelle's detailed description of bacteriophage destroying dysentery bacilli, many similar agents, capable of destroying a great variety of bacteria, have been isolated from various sources. Indeed, today the distribution of bacteriophages in nature is considered as widespread as that of bacteria. In fact, many investigators believe that it is very difficult to isolate bacteria from infected material without picking up an associated bacteriophage (154, 72). Phages acting against members of the colon-typhoid-dysentery group of bacteria have been the subject of most studies up to the present time; those capable of destroying staphylococci, however, have also been described frequently. Bacteriophages for members of the group of organisms represented by *B. mucosus capsulatus*, for plague bacilli, streptococci, thermophilic and psychrophilic bacteria have also been described. Recently Klosterman isolated a phage active against *B. diphtheriae*[3] and there are also single, as yet unconfirmed, reports of phages for meningococci and gonococci. No phages active against tubercle bacilli, pneumococci, and anaërobes have as yet been reported.

Questions naturally arise as to how such an ubiquitous agent could have been overlooked for so long by bacteriologists. Apparently it was not overlooked. When certain statements in the literature concerning bacteria growing with difficulty on ordinary media, cultures exhibiting marked tendency to autolysis, chromatolysis, or morphologic variations, and bacteria failing to be agglutinated by specific sera, are considered in the light of present knowledge of the changes in bacteria brought about by bacteriophage, it becomes evident that individual phases of the phenomenon of bacterial lysis have been repeatedly encountered in the past. However, these observations remained dis-

[2] Throughout this discussion, the terms bacteriophage, phage, and active agent will be used interchangeably when speaking of the phenomenon of transmissible lysis without reference to the nature of the agent. In referring to the hypothetical living virus of d'Herelle, the terms "Bacteriophagum intestinale" or "Protobios bacteriophagus" will be used.

[3] Klosterman, J. A., and Small, K. W., Recent studies on methods of isolating a bacteriophage for *Bacillus diphtheriae*, *Jour. Exper. Med.*, 1928, xlvii, 121. The phage isolated earlier by d'Herelle in 1918 (Technique de la recherche du microbe filtrant bactériophage (*Bacteriophagum intestinale*), *Compt. Rend. Soc. Biol.*, 1918, lxxxi, 1160) was active only against atoxic strains of *B. diphtheriae*.

connected until d'Herelle's work, which unified them in a single hypothesis.

Properties of Bacteriophage

Sedimentation.—According to d'Herelle, Bacteriophagum intestinale is corpuscular. This is indicated by the fact that on prolonged standing the active agent shows a tendency to settle to the bottom of the container. The rate of sedimentation is increased by centrifuging.[4] D'Herelle found that, after centrifuging for 30 minutes at a speed of 12,000 revolutions per minute, the concentration of bacteriophage in the bottom layer of the centrifuge tube was approximately 75 times greater than that of the surface layer (123—p. 280). These findings, however, have not received general confirmation.

Filtration and Ultrafiltration.—In his original description, Twort stated that the active agent present in the glassy material traverses Pasteur-Chamberland F and B, as well as Doulton-White filters (208). It has since been found that the phage as ordinarily obtained passes all siliceous filters without any detectable loss in its activity. Thus the pore size of all bacterial filters is apparently amply sufficient for the passage of bacteriophage. However, passage of colloidal particles through semipermeable membranes does not depend solely on the size of pores. Factors that affect force fields around the particles (both in the filtering fluid and in the filter), the surface tension at the zone of contact between the fluid and the filter, and the adsorption of the solvent on the surface of particles, are of paramount importance in determining the filterability of a colloidal solution.[5] It has been found, for instance, that bacteriophage acidified, or highly diluted with water or physiological salt solution, is partially retained by ordinary Berkefeld filters (61, 83, 90). On the other hand, basic filters of Kramer hold back also neutral solutions of bacteriophage (142). The fact that the behavior of the filtering surface may be radically influenced by conditions prevailing during filtration is probably responsible also for the widely divergent results obtained in ultrafiltration of bacteriophage. Thus, for instance, while some authors state that phage passes freely through collodion membranes which hold back enzymes and alkaloids (57, 207), others find that comparatively permeable membranes prepared from 2 per cent collodion hold it back (21, 210). Similarly, while Prausnitz

[4] The possibility of concentrating viruses by centrifugation has been questioned by Marshal, M. S., *Science*, 1927, lxvi, 219.

[5] This question is discussed in detail in the second chapter of this book.

(183) found that Haen membranes of medium permeability (100 to 200 seconds) hold back the bacteriophage quantitatively, Fränkel and Schultz (83) succeeded in filtering the phage through the least porous of these membranes, *i.e.*, the ones which held back all of the Congo red and some of the antisheep amboceptor.

Size.—On the basis of results obtained by ultrafiltration, several authors have estimated that the bacteriophage particles are approximately 20 $\mu\mu$ in diameter (21, 182, 207), and that each particle has a calculated mass of approximately $\frac{4}{3}\pi\ 10^{-18}$ gm. (184). Similar values were found by v. Angerer on the basis of his study of the turbidity of filtrates of lysed cultures (1). However, some investigators place the size of the bacteriophage particles considerably below (77) or above (22) 20 $\mu\mu$. The value of these conclusions may be questioned because, according to the author's findings, colloidal particles present in the lytic filtrates (and apparently endowed with the properties of bacteriophage) are not uniform in size and do not represent autonomous units of the active agent, but merely serve as a vehicle on which the active agent is adsorbed (46).

Adsorption.—Bacteriophage is readily adsorbed both by living and by dead susceptible bacteria. Exposure of susceptible bacteria to moist heat at 120°C. for 1 hour, however, destroys their power to bind phage (184). Heterologous or homologous resistant bacteria fail to bind appreciable quantities of phage (134, 162, 184). The active agent is readily adsorbed by various organic and inorganic colloids (38, 59, 71, 199), which explains its rapid disappearance from the circulation, following its experimental introduction into animals (199). There are no reliable data concerning the charge carried by the bacteriophage. It has been found by some workers to travel in the electric field toward the anode (1) and by others toward the cathode (141). Similarly, some authors have stated that it is adsorbed by positive, by negative, and by amphoteric colloids (156, 199). Others have found it to be adsorbed only by positive (90, 142), or by negative colloids (83, 184). It is evident that divergence in the findings is due to the failure to control experimental conditions. For instance, Gildemeister (90) finds that phage under ordinary conditions is adsorbed only by positive colloids. However, even a slight acidity of the solution makes it possible for kieselguhr to adsorb it. Another source of possible error is indicated by Yasaki (223), who finds that negatively charged kaolin does not remove phage from broth but does adsorb it from water. Similarly, negatively charged kieselguhr only adsorbs phage diluted by water (83).

Resistance of Phage to Physical and Chemical Agents

The question of the susceptibility of phage to physical and chemical injury has been the subject of repeated inquiries, because the early observations of d'Herelle led him to believe that the active agent of transmissible lysis behaves as a living organism and in general is somewhat less resistant to deleterious influences than are bacterial spores, but more resistant than are the vegetative forms of bacteria (115—p. 95). Unfortunately, in most instances, the investigators have not defined clearly the conditions under which their experiments were performed, and for this reason the task of comparing their results is often difficult.

Aging.—Workers generally agree that bacteria-free lytic filtrates keep well in the absence of evaporation. Not all the phages, however, are alike in this respect (123—p. 284); some (*coli*, dysentery) retain their activity practically unimpaired for years, others (staphylococcus, *pestis caviae*) show signs of appreciable deterioration after a few weeks.[6] In general, the rate of deterioration is increased with dilution.

Drying.—Although slow evaporation at room temperature reduces the activity of bacteriophage, some activity can be detected even after the fluid has reached the consistency of a syrupy mass. Similarly, bacteriophage is not destroyed by quick drying and pulverization (123—p. 287). There exist no exact data concerning the rate of deterioration of phage induced by drying.

Heat.—Findings concerning the resistance of bacteriophage to heat vary a great deal. The discrepancies in the results of different investigators apparently are due to the fact that in most instances they failed to take into account the composition, degree of dilution, and reaction of the medium in which the bacteriophage was exposed to the action of heat. Moreover, different "races" of bacteriophage exhibit various degrees of resistance, and d'Herelle has stated that even the age of the active agent affects its resistance to heat, inasmuch as the corpuscles of bacteriophage, according to him, undergo "condensation" and become more resistant to heat on aging (113). From the bulk of contradictory data, it appears that the exposure of undiluted phages, in broth of pH = $\pm$ 7.4 in sealed ampoules submerged in fluid, to a temperature of 70–75°C. suffices to inactivate them in from 30 to 60 minutes.

Cold.—According to recent investigations of Rivers (189), the effect of repeated freezing (–185°C.) and thawing on phage is comparable to

[6] Unpublished observations of Dr. Muckenfuss.

that on complement, trypsin, and colon bacilli. The partial deterioration of phage resulting from such treatment is influenced by the composition and concentration of the medium.

Reaction.—In the majority of statements regarding the effect of changes in hydrogen ion concentration on bacteriophage, the temperature or the length of exposure are not indicated. Taking into account these factors, the author found that different phages vary considerably in their resistance (39). Staphylococcus phage, for instance, was inactivated in 3 hours at 7°C. beyond the limits of pH = 4.3 and pH = 8.8, whereas *coli* phage under the same conditions was not inactivated until the reaction of pH = 1.55 or pH = 10.6 was reached. The phage for *B. pestis caviae* was found to be even more resistant to alkalinity and was inactivated only when the reaction reached pH = 2.9 or pH = 11.45, respectively.

Effect of Chemicals.—The question of resistance of phage to various chemicals has been studied more than any other single phase of this subject. The nature of chemicals used, the relative concentration of chemicals and phage, the temperature at which the exposures were made, and the composition of the medium in which the reaction took place vary to such an extent in the experiments of different investigators that it is impossible to bring together all the results in a brief review. In general, it appears that formalin and antiformin (each in the concentration of about 1 per cent) inactivate the phage in a few minutes. Alcohol (95 per cent) and quinine hydrochloride (1–3 per cent) require hours to accomplish inactivation. Other substances tested, *e. g.*, ether, acetone, chloroform, phenol (1 per cent), potassium cyanide (1 per cent), mercuric chloride (0.5 per cent), lysol, and glycerol, affect the phage more slowly than bacterial spores, and require days (2–30) to accomplish its complete inactivation.

It must be remembered, however, that in all these experiments the workers were dealing with preparations of phage in broth. The author's findings relating to the effect of alcohol on phage (40) suggest that, at least in this instance, the apparent inactivation of the lytic agent may be secondary to the changes in the vehicle on which it is adsorbed, inasmuch as comparatively pure phage (44) exposed to alcohol is much more resistant than is ordinarily supposed. As to the significance of such studies, recent investigations of Fabry (80) have shown that in many respects the behavior of phage toward chemicals is not essentially different from that of enzymes, on the one hand, and from that of vaccine virus, on the other.

Is the Bacteriophage an Organized Corpuscular Agent

A corpuscular nature is claimed for the phage by d'Herelle on the basis of the following observations. Filtrates of lysed cultures examined under an ultramicroscope show the presence of minute granules. Some of the granules can be seen entering the bacterial cells and multiplying within them (123—p. 273; 121—p. 255). The identification of these particles with the active agent is supported by the observation that on prolonged standing or as the result of intensive centrifugation of active filtrates, the particles tend to settle to the bottom of the container, and coincident with this settling, the active agent collects in the lower strata of the solution (123—p. 280). Furthermore, an active filtrate spread over the surface of agar seeded with susceptible bacteria produces discrete foci of lysis, and the number of such foci (plaques) is proportional to the concentration of the active agent in the solution (110). In addition to d'Herelle's observations, the author has found that the agent at high dilutions is distributed in the medium not as a true solute, but as particulate matter, inasmuch as it becomes at once totally ineffective as soon as dilution is carried beyond the point at which a certain quantum (about 1×10^{-10} cc.) of the agent is present (38).

Although the lytic agent is thus shown to be distributed in the medium in a particulate state, d'Herelle's conclusion as to the living nature of these particles does not seem warranted. It is not unreasonable to suppose that these particles are free molecules of some inanimate material, inasmuch as active filtrates consisting of phage, water, salts, tissue extractives in the broth, metabolic products of bacteria, and bacterial débris can be diluted 10^{10} times before reaching the limit of their activity. Furthermore, many observations indicate that the particles described by d'Herelle are not autonomous units of an active agent, but the vehicles on which the active principle is adsorbed and from which, under proper experimental conditions, it can be detached (46). For instance, if the particles carrying the phage are deposited on an ultrafilter, through which they cannot pass, and are washed repeatedly with water or buffer solution, they do not release any of the active agent. On the other hand, if the particles are washed with broth, the phage appears in the filtrate (46). Similarly, phage adsorbed on bacteria is not set free by washing the bacteria with water, but is liberated by washing them with broth (74). In addition, it has been shown that in a given volume of filtrate the number of particles endowed with the properties of phage can be altered by changing the composition

of the medium (38, 70, 166), *e. g.*, an increase in the amount of agar in the medium from 1 to 2.5 per cent suppresses 99 per cent of visible plaques (36). D'Herelle explains these results upon the ground that agar interferes with the diffusion of the metabolic products of the Bacteriophagum intestinale which in turn inhibit its growth (123—p. 89). Until it has been shown, however, that Bacteriophagum intestinale possesses an independent metabolism, this explanation of the phenomenon cannot be accepted.

Further evidence exists that the particles observed by d'Herelle (121) and others (21, 182) are not necessarily organized parasites. The lytic principle spreads radially from a focus on the surface of agar. This spreading is independent of the progress of "invasion" of bacteria, since it continues at 4°C.[7] (a temperature (115) at which the invasive activity of the phage is arrested), and since it occurs also in sterile agar (1, 7, 57, 185). If the autonomous particulate nature of the active principle be accepted, such spreading must be accounted for by postulating that the particles either possess the power of locomotion (135), or secrete a diffusible enzyme (115, 158, 188). Independent locomotion, however, would account for the transport of a given particle in only one direction at a time, whereas the active principle spreads from a focus in all directions simultaneously. The assumption that an active enzyme is secreted by Bacteriophagum intestinale is supported by inadequate evidence (38, 107, 123—p. 372). Moreover, it is difficult to correlate the facts concerning the experimentally produced changes (19, 36) in the size of the plaques with d'Herelle's idea that the plaques are "colonies" of bacteriophage (123—p. 87). For instance, if the size of a plaque depends on the progressive invasion of bacteria by the parasite, it should be directly proportional to the number of susceptible bacteria around the initial particle of Bacteriophagum intestinale. As a matter of fact, however, the concentration of susceptible bacteria in the immediate vicinity of the original particle and the size of plaque formed vary inversely (38), provided other conditions remain constant. It is also difficult to explain on the basis of d'Herelle's hypothesis, why there is a

[7] Unpublished experiments of the writer in collaboration with Dr. Korb in 1924. Agar containing phage and bacteria was poured into plates and incubated at 37°C. As soon as the formation of plaques became visible, the plates were placed overnight at 4°C. The next day they were returned to the incubator, and when the growth of bacteria was resumed (at 37°C.), a halo of lysis appeared around the original plaques, indicating that phage had diffused during the night beyond the periphery of the original plaques (Fig. 1).

decrease in the number and size of plaques coincident with an increase in the number of *old* bacteria which are not subject to lysis, whereas an increase in the concentration of *resistant* bacteria affects neither the number nor the size of plaques (38). A plausible explanation of these phenomena is the one based on the fact that the lytic agent tends to be adsorbed on agar and on old susceptible bacteria, but not on resistant organisms (140, 162). The amount of phage adsorbed under different conditions naturally influences the amount left in a diffusible state and consequently the size of the plaques also.

Antigenic Properties of Bacteriophage

In spite of the evidence that the particles observed may not be autonomous parasites but the vehicles on which an active agent is adsorbed, the fact still remains that the active agent itself may be particulate, that is, may consist of minute aggregates, each of which is composed of a number of molecules. Furthermore, these small aggregates, probably much less than 20 $\mu\mu$ in diameter, may still possess all the characteristics previously ascribed to the larger carrier-particles and may still represent d'Herelle's "colloidal micellae which differ from other forms of life only in their size" (124). The colloidal nature of the active agent is suggested by its behavior with respect to solubility. The dried preparations of bacteriophage cannot be dispersed as completely as they were originally. This is particularly true of preparations of bacteriophage deprived of all, or nearly all extraneous material (44). The colloidal nature of the active agent is further indicated by the fact that it is antigenic, and produces specific antibodies when it is introduced parenterally into experimental animals (9, 28, 172, 204, 213). While the antiphage sera thus obtained usually contain also agglutinins, opsonins, precipitins, and complement-fixing antibodies for homologous bacteria, (14, 216) and while purely antibacterial sera are apparently able to inhibit lysis of bacteria by homologous bacteriophages (14, 63, 108, 157), it has been possible, nevertheless, to establish the fact that the bacteriophage is an independent antigenic entity. When sera prepared by means of active filtrates are adsorbed with homologous bacteria, they no longer react with bacteria and their products, but retain their full power to neutralize the bacteriophage (63, 185, 216). Furthermore, antisera obtained by immunizing animals with preparations of purified phage neutralize the active agent without reacting with bacteria (6, 27). Moreover, in the case of phages capable of acting on several related species of bacteria, the antigenic specificity of the filtrate is constant for each phage and is

independent of the bacterial substratum employed in obtaining the active filtrates (53, 55, 200, 211). Finally, it has been demonstrated that the phage-inhibiting activity of antibacterial sera is not due to effects of these sera on the phage, but to their action on the bacteria rendering them less susceptible to lysis. It has been suggested that this effect is due probably to the saturation by agglutinins of receptors susceptible to union with phage (62, 157).

Mode of Action of Antibacteriophage Sera

According to Bordet, the active agent is destroyed by antiserum. If the phage is an organized corpuscular virus, one might expect, in view of what is known about the mode of action of bactericidal sera, that complement would be necessary for a virucidal action of antibacteriophage sera. The results of the author's experiments, however, have shown,[8] in accord with earlier findings of Bail (14), that heated antibacteriophage serum is as effective in inactivating phage as is the same serum previous to destruction of its complement. With the exception of Osumi (172), a number of workers have since come to similar conclusions (200, 211, 213) and suggest that the mode of inactivation of the bacteriophage by specific antisera is analogous to the manner in which toxin is neutralized by antitoxin, inasmuch as the inactivation occurs according to the law of multiple proportions, and also because after complete inactivation the union between phage and antibodies can be broken by various procedures, releasing phage and antibodies in an active state (117, 200, 217).

Metabolism of Bacteriophage

The fact that a portion of the bacterial substratum is gradually transformed into an antigenically distinct substratum by the bacteriophage indicates to d'Herelle that the phage is alive and "must" possess an autonomous metabolism (122, 188). In the absence of direct evidence of such metabolism, he relies for support of his idea concerning this matter upon indirect evidence supplied by Wollman and by Asheshov, namely, that "multiplication of bacteriophage corpuscles can take place, at least to a certain degree, also in the absence of the bacterial cells, and that for this development the corpuscles utilize certain diffusible products present in the culture of susceptible bacteria" (123—pp. 102 and 103). However, the findings of Wollman (220), which he himself ques-

[8] Unpublished experiments in coöperation with Dr. Korb, 1923.

tioned later (221), can be adequately interpreted in a manner different from that used by d'Herelle, and in the light of results obtained by the writer (47) do not indicate that the bacteriophage assimilates any products of bacteria. Jötten (134) reported that phage can multiply in the presence of dead susceptible bacteria alone. This work, however, has not been confirmed (47, 58, 176, 199). The recent work of Twort, showing that dead bacteria may undergo lysis, if, in addition to bacteriophage, living homologous bacteria are present, seems to have no direct bearing on this question. Even if the lysis of dead bacteria were due directly to the lytic activity of the phage (this was shown by the writer (47) not to be the case), so long as living bacteria are present, the increase of phage cannot be attributed directly to the assimilation of dead bacteria by the phage.

Apart from the nature of the substratum supposedly utilized by the Bacteriophagum intestinale, Asheshov attempted to demonstrate the existence in the phage of an autonomous metabolism by "vaccinating" animals and susceptible bacteria with "ultra-sterile" filtrates,[9] presumably free from bacteriophage, yet containing the products of its metabolic activity (12, 13). In his experiments, as in those quoted above (123—pp. 102 and 103), the plaque method for determining the "ultra-sterility" (absence of active phage) of the filtrates was employed. It is conceivable that he might have overlooked the presence of a small amount of phage in the filtrate, inasmuch as phage which has been put through a collodion membrane tends to yield extremely small plaques (38, 46—p. 880, footnote). This assumption is strengthened by the observations of Wollman, who attributes his own erroneous conclusion (220) concerning the impermeability of collodion to phage to the failure to detect by the plaque method minute amounts of the active agent present in the filtrate (221). In view of these considerations, the results of Asheshov's vaccination experiments are probably due to the presence in his "ultra-sterile" filtrates of small amounts of active phage, and cannot, without further evidence, be considered as proof that the Bacteriophagum intestinale has an independent metabolism.

The question of independent metabolic activity on the part of bacteriophage has been approached also from other angles. By using a specially constructed microrespirometer, the writer was unable to detect CO_2 production by 10^{12} active units of bacteriophage during a period of 96 hours (35, 43, 45). Due to the fact that the method does not reliably

[9] These "ultra-sterile" solutions were obtained by filtration of phage through collodion.

measure less than 0.005 cc. of CO_2, there still remains the possibility that the agent does respire but that during the experiment the amount of CO_2 produced was too small to be detected.[10] However, when the Bacteriophagum intestinale is actively "multiplying" in cultures of growing susceptible bacteria, the rate of respiration of the whole culture, corrected for the higher rate of multiplication of bacteria in the presence of phage, was found to be the same as that of a control culture without phage.[11] For the sake of argument, however, suppose bacteriophage does respire. It has been calculated that its respiration must be at least 10,000 times slower than that of an equal number (10^{12}) of germinating spores in order not to be detected in 96 hours. It would be expected *a priori* that the rate of the respiration of phage would be greater than that of spores, since it is generally observed that the smaller the living organism, and consequently the greater its surface per gram of its mass, the higher is its rate of respiration. Since the size of a "corpuscle" of Bacteriophagum intestinale is but a small fraction of the size of a bacterial spore, the finding that the rate of respiration of the unit (quantum) of phage is at least 10,000 times less than that of the spore seems to indicate that the phage probably does not respire at all. More recently, by using a different type of apparatus Bachmann and Wohlfeil (*Cent. Bakt., Abt. I, Orig.*, 1927, civ, 256) also came to the conclusion that bacteriophage does not respire.

Conclusions similar to those above were reached by several investigators who studied the independent metabolism of phage as expressed in terms of its reducing power. The findings of Fejgin (82), Gózony and Surányi (94), Kauffmann (137), Shwartzman (197), as well as those of the writer[12] indicate that the rate of reduction exhibited by cultures of susceptible bacteria grown in the presence of bacteriophage is independent of the concentration of the active agent, and rises and falls with the number of intact bacteria present during the various phases of growth and lysis.

The consensus of opinion at present is that the increase in the amount of active phage occurs *exclusively* in the presence of living, actively multiplying bacteria, and that the question as to whether the phage has an independent metabolism of its own still remains an open one.

[10] Since the last published report and after a further improvement of the apparatus, the experiments were carried over periods of 10 days without detection of CO_2 production by the phage.

[11] Unpublished experiments in collaboration with Dr. Reichert, 1925.

[12] Unpublished experiments (1925) in collaboration with Dr. Reichert.

Adaptation of Phage to Injury by Chemical and Physical Agents

Evidence obtained from experiments dealing with the effects of various physical and chemical agents upon phage has been used for and against the idea that the lytic agent is an animate parasite (113, 115, 184). A critical analysis of the results of these experiments, however, reveals no single test that may be used as a criterion of the presence of life (189).

A certain degree of apparent adaptation on the part of Bacteriophagum intestinale to the deleterious effects of some chemicals has been reported by several workers and adduced by them as proof of the living nature of phage. The results of d'Herelle's experiments with glycerol (115—p. 95) are not convincing and may be analogous to the phenomena observed when arsenic sulphide is coagulated by acid, *e. g.*, the amount of acid necessary for coagulation is greater when the acid is added gradually than when the full amount is added at once. The results obtained by Prausnitz (184), who observed the adaptation of phage to phenol, mercuric chloride, and immune serum, were confirmed by Munter and Rasch (164) only when they used Prausnitz' phage as well as his strain of *B. dysenteriae*. Their findings in this one instance were considered by them to be due not to the ability of the phage to adapt itself to adverse conditions, but to the lysogenic character of the strain of bacteria employed. The observation of Asheshov in regard to the adaptation of phage to gradually increasing hydrogen ion concentration (10, 11) are of little significance, inasmuch as he himself admitted that such an adaptation occurred with only one specimen of phage.

Apart from the possibility that many of the above results may have been erroneously interpreted, the significance of the findings may be questioned for another reason, namely, that the observed increase in the degree of tolerance supposedly due to adaptation was usually so small as to be negligible. This is particularly significant inasmuch as the workers themselves admit elsewhere (11, 184) that a given sample of phage, tested at different times, frequently exhibits a marked variability in its behavior.[13]

[13] In discussing the attempts of investigators to interpret variations in the behavior of different samples of phage as indicating the existence of many independent and immutable phages, as opposed to his concept of "unicity" of the Bacteriophagum intestinale, d'Herelle says: "As for variability in resistance [of phages], clearly if the resistance of each race were a fixed property, this argument would deserve consideration. But this is not what is observed. The studies of all authors who have considered this question, even including the collaborators of Bruynoghe, show that from one experiment to another a single bacteriophage presents considerable variations,—differences as great as are those observed between the different races" (123—p. 368).

Is There Only One Bacteriophage

The question as to whether there is one or more phages has caused considerable discussion. In spite of the experiments discussed in the previous sections, the majority of workers believes in the existence of relatively constant and immutable differences in the response of various phages to environmental conditions. Indeed, many investigators have used these individual characteristics as a means of identification and even separation of single phages from mixtures containing several active agents (53, 126, 188). The fact that the characteristics of individual phages are significant and are not due to inconsequential variations, has been demonstrated not only with respect to resistance to chemical (39, 51, 53, 211) and physical agents (53, 99, 172, 211), to size and character of plaques (53, 126, 161, 171, 211), but particularly in connection with the strict serologic specificity existing even among closely related phages (16, 52, 53, 99, 200, 211). This antigenic specificity, as stated earlier, is quite characteristic for each phage, and in the case of polyvalent phages[14] is independent of the nature of the bacterial substratum used in their production (53, 200, 211). D'Herelle's conclusions in regard to the serologic identity of different "races" of Bacteriophagum intestinale were based on results obtained by means of complement fixation tests. His results, however, have since been shown to be due to a non-specific fixation of complement by the rabbit serum used by him as a source of antibody (86, 191, 194).

It has been shown repeatedly that the secondary growth of bacteria which appears after the completion of lysis of susceptible bacteria by a given bacteriophage, is specifically resistant to this phage, but is often susceptible to lysis by some of the closely related varieties (17, 53, 99, 126, 159, 161). According to d'Herelle, this phenomenon does not prove the plurality of phages, inasmuch as he observed that *B. typhosus* grown in the presence of a single polyvalent phage yielded resistant forms which, in turn, were susceptible to the lytic action of filtrates prepared from *B. coli* and *B. dysenteriae* grown in the presence of the same polyvalent phage. The writer, however, using a polyvalent phage similar to d'Herelle's was unable to confirm his findings.[15]

A certain degree of species specificity is observed in the action of the lytic agents. Samples of phage recovered from convalescent human

[14] The term "polyvalent phage" signifies a single phage capable of acting upon several kinds of bacteria.

[15] Unpublished experiments in collaboration with Dr. Muckenfuss, 1926.

beings and animals are in the majority of instances active exclusively against the species of bacteria which produced the infections. At times, however, not only recently isolated phages, but also purified ones, act on several and sometimes unrelated bacteria (123—p. 366). The fact that some strains of phage are lytic for different species of bacteria indicates to d'Herelle that "there is but a single bacteriophage, common to both man and animals, capable by adaptation of acquiring a virulence towards all bacterial species" (119—p. 121). While several authors claim to have observed such a transformation of phage (2, 132, 165, 222), others have found that this phenomenon is by no means frequent (176). Furthermore, many investigators have been unable to experimentally induce such an adaptation of phage, even when they used closely related species of bacteria (17, 79, 158, 160). It has been suggested that, in the instances in which an adaptation apparently occurred, the workers were dealing with polyvalent phages possessing from the start a latent activity toward several bacterial species that became evident upon repeated passage in cultures of the various bacteria (100, 198). Another plausible explanation of the apparent adaptation is to be found in the possibility that the lytic filtrates used in the experiments were mixtures of several independent phages (17, 159). This seems not unlikely in view of the fact that several investigators, who apparently succeeded in securing an adaptation of phages to new species of bacteria, observed that the activity of the phages for the original species of bacteria was greatly weakened or lost (probably through dilution) during the process of adaptation (2, 132, 218, 222).[16]

Relation of Lysis to Production of Phage

The dissolution of bacteria and the multiplication of the active agent causing it, originally centered Twort's attention on the phenomenon of transmissible lysis. Although in his original publication Twort (208) described most of the properties of the active agent, he made no definite statement concerning its origin, nature, or mode of action. It is apparent, however, that he considered the disintegration of the bacteria as the significant feature of the process. Indeed, the expressed views of many workers, including d'Herelle, suggest that the lysis of bacteria is the essential part of bacteriophagy, and that the accumulation of phage

[16] By mixing two independent phages (*coli* phage and staphylococcus phage) the author has been able to imitate this phenomenon of apparent adaptation of one of the phages and gradual elimination of the other, depending on the species of bacteria used for successive passages.

depends entirely on its liberation through dissolution of bacteria (123—p. 74).

If the above view is correct, there should be no accumulation of phage without lysis, and, conversely, lysis should always be accompanied by an increase of the concentration of the active agent in the medium. A number of observers, however, have brought forward evidence showing that such is not the case. This evidence was obtained by determining simultaneously, at stated intervals, the amount of phage and the number of bacteria per cubic centimeter in given cultures under experimental conditions. These experiments indicated that, in the presence of undiluted phage, the susceptible bacteria undergo complete lysis without increasing the concentration of the phage in the medium (18, 161, 163). On the other hand, if the initial concentration of the phage in the medium is low, one can detect the production of more phage long before any lysis of bacteria sets in (68, 192).[17] Moreover, under special experimental conditions visible lysis can be prevented entirely in spite of the normal regeneration of the phage to its maximum (51, 60, 62, 69, 161, 175, 192, 225).

According to the findings above, it appears that the phage, far from being the product of lysis, is regenerated during the stage of active multiplication of susceptible bacteria preceding the lysis. This fact is further emphasized by the following observations. If the multiplication of bacteria does not proceed at a normal rate, either due to the age of the culture or as a result of special experimental conditions, the rate of accumulation of the active agent may not only be reduced materially (54, 68, 71, 111, 177, 196, 225), but its concentration in the culture medium may actually be diminished through adsorption on bacteria (71, 177). Such is the case, for instance, when cultures of ordinary bacteria containing the phage are incubated at 43–45°C. instead of at 37°C. (71, 145).[18]

That the failure in production of new phage at this temperature is due to changes in bacteria, and not in the agent itself, can be inferred from the fact that phage is comparatively resistant to heat, and shows no deterioration until the temperature is raised at least above 60°C. Moreover, it has been found that the bacteriophage regenerates in a normal manner even at a temperature higher than 45°C. and lower than 8°C.

[17] Similar observations were made also by Dr. Muckenfuss in the author's laboratory.

[18] According to d'Herelle this phenomenon is not general and can be reproduced only with certain strains of phage (123—p. 61).

if the susceptible bacteria can multiply normally under such conditions. Thus the phage for thermophilic bacteria regenerates best at the temperature ranging from 45°C. to 52°C. (143). At 37°C. the growth of these bacteria is retarded and the regeneration of phage is correspondingly less rapid and less complete. Similarly, in the presence of psychrophilic bacteria, which multiply best at a temperature between 0.05° to 4°C., the regeneration of phage is also best between 0.05° to 4°C., but does not occur at 37°C., a temperature at which the growth of the psychrophilic bacteria is arrested (78). Thus, one may conclude that the regeneration of phage takes place only so long as conditions permit normal multiplication of susceptible bacteria,[19] and that lysis of bacteria is merely a secondary phenomenon which may or may not follow the accumulation of phage.

Origin of Bacteriophage

The fact that the accumulation of phage in the medium is independent of bacterial dissolution and is intimately associated with the active growth of bacteria, strengthens the view expressed earlier that the active agent may originate as some product of bacterial metabolism. In order to obtain a new strain of bacteriophage, one usually introduces into a culture of bacteria some material contaminated more or less directly with intestinal contents of higher animals. The nature of this material is such that the possibility of the presence within it of some living ultramicroscopic agent cannot be excluded. Therefore, if one were able to bring about the appearance and accumulation of the agent of transmissible lysis in cultures of bacteria by such means that would exclude every possibility of the introduction of an extraneous living agent, the bacterial origin of phage would be definitely established.

A number of investigators claim to have demonstrated in a variety of ways that phage originates from bacteria. Their experiments, however, are not free from the criticism that either the material used to induce the appearance of phage, or the bacterial cultures themselves, might not have been actually free from phage. From what is known about the wide distribution of phage in the animal body (84, 118, 120, 129, 181, 219), it is quite evident that an active agent obtained by introducing bacteria into the peritoneal cavity of guinea pigs (23, 25, 26, 176), or by

[19] The experiments of Wollman (220) and Jötten (134) which suggest that regeneration of phage may take place in the absence of actively multiplying bacteria do not invalidate this conclusion. These experiments were fully discussed in an earlier part of this chapter.

exposing bacteria *in vitro* to the action of leucocytes, tissue extracts, body fluids, and ferments (24, 25, 57, 97, 134, 146, 150, 155, 179, 180, 187), cannot be considered as proof of a "spontaneous" appearance of the active agent. All such substances may conceivably be contaminated with phage.[20] Furthermore, it is known that many strains of bacteria (particularly those recently isolated) frequently carry phage (72, 154) which cannot always be demonstrated with ease. This difficulty probably arises from the fact that only a few bacteria in the culture may be susceptible to the action of this phage. Thus the activity of a phage may remain latent for a time in a given culture and then suddenly become manifest if the susceptibility of the population of the culture is suddenly changed, or if the filtrates of such "lysogenic" cultures are allowed to act upon more susceptible strains of homologous and occasionally even of heterologous bacteria. This "lysogenic" power of some cultures manifesting itself against other homologous and heterologous bacteria has been adduced as proof that the phage may arise "spontaneously" as a result of bacterial antibiosis (30, 91, 92, 151, 152). The writer, however, has been able to imitate experimentally this phenomenon in every detail. By subjecting a phage-free culture of colon bacillus to the action of a polyvalent phage and then isolating the overgrowth of resistant bacteria, it was found that cultures of these resistant forms carried the phage for months without showing any signs of its presence.[21] When, however, these resistant cultures were grown in symbiosis with stock cultures of *B. coli* or *B. dysenteriae* Shiga, the presence of phage was readily demonstrated.[22]

In view of the fact that cultures of bacteria may thus carry phage without revealing any signs of its presence, it is advisable to be very cautious in interpreting the sudden appearance of phage in cultures

[20] The only promising experiments of this nature have been reported by Doerr (71), who obtained bacteriophage by feeding susceptible bacteria to sterilely hatched chickens. These experiments have not been repeated, however, and it is impossible to say at present whether the result was merely an accidental occurrence. Somewhat similar experiments were performed by Ebeling in collaboration with the writer without obtaining positive results in the preliminary trials. In these experiments bacteria were introduced into pure cultures of cells of intestinal mucosa from chick embryos.

[21] A few susceptible bacteria present among the mass of resistant ones apparently permitted very slight regeneration of phage to take place at each transfer, thus securing conditions perpetuating the resistance of the majority of bacteria in the culture.

[22] Unpublished experiments in collaboration with Dr. Muckenfuss, 1927.

subjected to various influences of physical or chemical nature, or even in cultures merely kept in the laboratory for a long period of time (23, 88, 89, 134, 169, 177). Quite apart from the possibility of an occasional direct contamination of these cultures with phage present in the laboratory, such manipulations as, for example, filtration through Berkefeld filters (174, 177, 187, 202, 214, 215), a usual step in the preparation of bacteriophage, have been known to result in contamination of filtrates with phage remaining on the filter from previous experiments and not completely removed in washing and sterilization of the candles (186, 201). Furthermore, the "spontaneous" appearance of phage incited by adding to a bacterial culture various chemicals (31, 133, 174, 177, 178) (including water (177, 214)) may conceivably be the result of bringing to light a latent phage already existing in the culture through the production of a selected bacterial population, which may occasionally happen to be more susceptible to the phage than was the original culture. No matter how remote the possibility of making manifest a latent phage may seem to be, it should always be kept in mind, inasmuch as the investigators who were successful in producing the "spontaneous" appearance of phage were not able to obtain positive results with every culture, nor were they able to repeat them consistently with the same culture (85, 176, 185). Others were never successful in their attempts to reproduce this phenomenon (24). During the period of the last 4 years, the writer has attempted repeatedly, by various procedures, to induce the "spontaneous" appearance of phage in a variety of cultures known to be free from phage. Thus far, the results have been consistently negative. Most recently, however, Fukuda (*Z. Immunitätsforsch.*, 1928, liv, 369) reports having succeeded in inducing spontaneous production of phage in such cultures of known purity.

Although facts concerning the phenomenon of bacteriophagy leave an impression that the active agent of transmissible lysis is a bacterial product, the reports concerning the successful production of phage from bacteria without the intermediary of other biologic materials have thus far not been entirely convincing.

The Mechanism of Clearing of Cultures by Phage

The results of investigations reviewed in the preceding pages indicate that d'Herelle's conception concerning the phage has not been substantiated. Although the agent, probably a colloid, represents an autonomous antigenic entity, it cannot be identified with the particles which apparently carry it. The agent exhibits no metabolic activity

when studied in the absence of living susceptible bacteria, the metabolic rate of mixtures of phage and bacteria depends entirely on the number of living bacteria in the mixtures, and not on the concentration of the agent. The evidence that the agent is capable of adapting itself to environmental changes is not convincing. The fact that the regeneration of the active agent depends wholly on the presence of living, susceptible bacteria suggests that it may be a product of some phase of bacterial activity. Indeed, several investigators have reported that they were successful in obtaining phage directly from bacteria. Their results, however, are not free from possible criticism. At all events, it is clear that when traces of active agent are introduced into a culture of growing susceptible bacteria, the regeneration of the agent takes place during the phase of active growth, and precedes the lysis of the bacteria (when the latter takes place at all). Thus, the possibility that the active agent is liberated as a result of lysis of bacteria seems to be excluded.

Concerning the actual nature of the active substance and the mechanism of its production by bacteria, a great divergence of opinion exists, and, as yet, no definite evidence has been offered to substantiate any of the opinions expressed (15, 25, 66, 73, 105, 136, 167, 177, 190). However, since the active agent is comparatively stable under ordinary conditions, hope still exists that it may be isolated in a pure state and that its nature may then be determined.[23]

As to the nature of the effect of phage on normal bacteria, resulting on the one hand in its own regeneration, and on the other in the disappearance of visible bacterial growth, there exist two views. The majority of workers think that the effect consists in a vitiation of bacterial metabolism resulting in an accumulation of products which under normal conditions either are not formed, or appear only temporarily as intermediary links in the chain of metabolic reactions of bacteria. The accumulation of these products in turn causes disfunction of new generations of bacteria, and thus perpetuates the disease leading finally to autolysis of bacteria.[24] Thus far, however, the identity of the products

[23] Some of the experiments now in progress in the writer's laboratory indicate that purification of this agent can be carried to a point where it no longer gives any of the protein tests, though its activity and its antigenic properties remain unimpaired (44).

[24] According to some investigators, the products of this vitiated metabolism of bacteria possess enzymatic properties themselves (106, 146, 177, 180, 182, 192) or are capable of acting as catalysts activating the normal intracellular proferments of the bacteria (58, 136). The relative specificity of phage action is ex-

actually accumulating in the culture with those assumed by different workers to be present has not been proved. There is also no explanation of the mechanism responsible for the sudden onset and extremely rapid progress of bacterial dissolution. Besides, reported chemical analyses of cultures, after completion of lysis, have failed to indicate any increase in protein-split products which would be expected to take place if bacteria were autolyzed (132, 216, 225). The opinion of another group of workers is that the dissolution of bacteria is not due to hydrolysis, but is the result either of a simple plasmolysis (132) or of a special, highly hypothetical type of cleavage not involving chemical disintegration of bacterial cells.[25] These workers offer no experimental evidence in support of their views.

Some light as to the nature of the changes actually taking place in the bacteria under the influence of the active agent seems to come from the direct observation of lysis under the microscope. As has been mentioned earlier, d'Herelle observed that soon after the exposure of bacteria

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plained by them on the basis of Salkowski's findings that ferments in general (and proteolytic ferments in particular) are often most active against the constituents of such cells which originally produced them (193).

According to others, they resemble growth hormones (66), or exhibit cytotoxic properties (163), and increase the rate of metabolic activities of bacteria to such an extent that lysis occurs as a terminal stage of this trophic disease.

Again, it has been suggested that with the abnormally rapid rate of growth, the amount of proteolytic enzyme set free by the bacteria is so great that supply of protein is exhausted and the ferments attack bacteria themselves (203).

According to Bail (20), autolysis of bacteria is due to the loss by the bacterial chromosomes of their anabolic function. The cells with imperfect chromosomes, possessing only the function of katabolism, undergo dissolution. The imperfect chromosomes are thereby set free, and are capable of initiating similar vitiation in other bacteria.

Another group of workers suggest that the abnormality in bacterial development leading to autolysis is transmitted from generation to generation by heredity (25, 221) or is perpetuated through a continuous effect of bacterial dissociation which results in the tendency of the culture to give off increasing numbers of nonviable, autolyzing variants (4, 73, 127).

[25] Some workers postulate the existence of a complex life cycle in the development of bacteria, during which bacteria may give rise either to functionally incomplete and not self-perpetuating elements ("spermites" (105) or "ultraspores" (190) or "schezomeres" (Kuhn, P., *Beihft. Arch. Schiffs-u. Tropen-Hyg.*, 1926, xxx, 133) which possess all the properties of bacteriophage. Others believe that bacteria may give rise to living, filterable fragments which are capable of perpetuating this transformation by causing abnormally rapid growth of the culture either directly (167) or through the intermediary of ferments secreted by them (87).

to bacteriophage, the former begin to swell and finally burst, due to internal pressure which he thought was exerted by the parasites multiplying within the cells (116, 123). While the swelling of bacteria under the influence of phage has been repeatedly observed by others, its constancy or its importance as a factor in the disappearance of bacteria has not been generally accepted (58, 60, 109, 154, 185, 209, 221, 225). The impression gained by several workers from observation of fresh as well as of stained preparations is that only comparatively few bacteria undergo swelling, and that the great majority of bacteria disappears without undergoing any visible change (58, 64, 154, 185). Moreover, it has been suggested that swollen bacteria do not finally burst (58, 221), but that they are particularly resistant and disappear, if at all, very slowly (58, 64, 93, 185).

The extent of swelling of individual bacteria, the relative proportion of swollen cells, as well as the actual relation between the swelling and the lysis, are difficult to establish by direct observation alone; the swelling and the lysis go on simultaneously and continuously, and at different rates in the case of different bacteria in the same culture (Figs. 2–3). Moreover, the actual disappearance of individual bacteria from the field of observation in fresh preparations is so sudden and rapid that it is difficult to ascertain accurately the morphological appearance of each bacterium just prior to its disappearance. It is evident that such direct observations must lead to highly divergent conclusions when carried out by different investigators, and even by the same investigator at different times (185).

The writer thought that an objective index of degree of swelling, and establishment of relation of swelling to lysis, might be obtained from a study of changes in the relative volume occupied by the entire bacterial population as expressed in the viscosity of the culture (42). These studies have indicated that in about 1 or 2 hours[26] after the addition of bacteriophage to the heavy suspension of susceptible bacteria, the viscosity of the mixture begins to increase and slowly progresses up to the time just previous to lysis, at which period it reaches its maximum. When the process of clearing sets in, the viscosity of the mixture also commences to diminish and in a comparatively short time it returns to the initial level. As controls, in place of susceptible bacteria, either cultures of homologous resistant strains or of heterologous bacteria, or suspensions of susceptible heat-killed bacteria, were employed. In

[26] This interval varies with the concentration of phage.

another series of experiments conducted as an additional control heated phage was used in place of the active preparation. As calculated on the basis of viscosity changes (144), the entire bacterial mass occupies, at the time of maximum swelling, from six to twelve (or more) times the volume occupied by a similar number of normal bacteria. The fact that, following visible clearing, the viscosity of the mixture reaches approximately its original level, seems to indicate that swollen bacteria are the ones which eventually disappear.

This conclusion was substantiated by a cinematographic record of lysis of *B. coli* on agar, at 37°C. (48).[27] As the illustrations show, swollen bacteria with perfect outlines in one photograph are absent in the next taken less than 3 seconds later (Figs. 5 and 6). Usually in the space formerly occupied by a bacterium, there remains nothing but the faintest trace of amorphous débris, which, in turn, disappears in less than 1 minute, leaving only a few more or less refractive granules (Fig. 5). Occasionally the débris left after the disappearance of the bacteria is less easily soluble, and is recorded on the film for a longer period of time (Fig. 6). This happens usually in the case of cells which disappear after having undergone only moderate swelling. Markedly swollen cells leave practically no trace after their sudden disappearance.

The rapid melting away of the bulk of the cytoplasm, which under ordinary circumstances is supposed to be semigelatinous in consistency, suggests that it probably is liquefied within the cell prior to the disappearance of the membrane. Stained preparations also suggest that the cytoplasm is liquefied prior to the rupture of the cell membrane. In swollen bacteria stained by Gutstein's method (102), the cytoplasm may be differentiated from the ectoplasm. The latter always appears continuous, and even in extremely distended cells it shows no evidence

[27] The progress of lysis was photographed by Mr. Rosenberger, by means of an automatic camera at a rate of 25 exposures per minute. The record shows that, after a short period of lag, bacteria began to multiply at a rate noticeably exceeding that of normal bacteria photographed under similar conditions, but in the absence of phage. This stimulating effect of phage has been reported by a number of investigators. Due to the rapidity of growth, many cells failed to divide completely and gave rise to filaments having a length of from 10 to 20 times that of an average normal cell (Fig. 2). By the end of the first hour of growth, occasional cells began to show signs of swelling, and by the end of the third hour the great majority of cells in the field appeared more or less swollen, a few among them reaching several times their normal dimensions. Filaments swelled as well as ordinary bacteria (Fig. 4). The swelling continued slowly until about the fifth hour, when, one by one, bacteria began to disappear suddenly and quickly. Some of the bacteria disappeared when only moderately swollen (Fig. 6).

of the "holes" described by d'Herelle (123—p. 115) as resulting from the puncturing of the membrane by entering parasites. The cytoplasm, on the contrary, shows marked changes during swelling. It takes the stain less intensely and less evenly as the swelling progresses, so that in many instances it appears segmented or beaded[28] (Fig. 7). In unstained cells photographed at this stage by means of ultraviolet light,[29] the cytoplasm appears to be of uneven density, quite unlike that of normal bacteria. Some of the stained preparations of highly swollen bacteria suggest that the material which normally binds the chromatin has been digested, and that the chromatin in the form of dust-like particles has been distributed throughout the cytoplasm (Fig. 4).

If the observations made during these experiments are correct, one can hardly fail to conclude that the cytoplasm underwent digestion during the progress of swelling, so that when it was set free, following the disappearance of the cell wall, it immediately diffused throughout the medium. If such were the case, however, chemical examination of the medium should show the presence of products of digestion of the cytoplasm. As stated previously, repeated analyses of lysed cultures have failed to show the presence of such products (132, 216, 225). The failure of these attempts to detect hydrolysis might have been due, however, to the fact that the material subjected to analysis consisted of ordinary culture medium too rich in various products of protein hydrolysis to permit the detection of a possible small increase due to the hydrolysis of bacteria. Our experiments in which lysis of bacteria occurred in a synthetic medium, devoid of all protein, gave unmistakable evidence of hydrolysis of bacterial protein (48, 49).

In the light of the above experiments, the clearing of bacterial cultures in the presence of bacteriophage seems to be due to hydrolysis of bacteria. The active agent (bacteriophage) plays no part in the actual dissolution, which is the result of intracellular digestion brought about by normal endoferments. Just how these endoferments become activated is at present unknown.[30] The increase of osmotic pressure within the cells, following the beginning of digestion of the cytoplasm, leads to an imbibition of water, which probably facilitates further digestion (131,

[28] Somewhat similar changes were also brought out by the toluidin staining by v. Preisz (185).

[29] These photographs were taken by Dr. F. L. Gates.

[30] It has been suggested by Warburg (212), however, that profound changes may be initiated in a living cell by toxic substances, even in cases where these substances do not penetrate the cell but act only on the membrane.

168) and progressive swelling. The degree of swelling of individual bacteria depends on the ability of the membrane to withstand the pressure. When the limit of turgescence of a given cell membrane is reached, it ruptures and the predigested intracellular contents diffuse throughout the medium.

The phenomenon of transmissible lysis can thus be divided into two stages. The early stage depends on the presence of the phage, which stimulates bacteria. As a result, the activity of intracellular ferments is increased, and the rate of multiplication is exaggerated.[31] Some of the products, bacteriophage, escape from the cells[32] into the medium, and in turn produce a stimulating effect on newly formed cells.[33] They are toxic only to closely related species of bacteria, hence the multiplicity and relative specificity of different bacteriophages.[34] The second stage of the process, which may or may not follow the first depending on circumstances, consists in the swelling and eventual bursting of the bacterial cells due to the intake of water following the increase in osmotic pressure within the cells. Any change in environment which tends to interfere with the progress of intracellular digestion or with the freedom of passage of water from the medium into the cells, may prevent the swelling and lysis of the bacteria. This probably explains the inhibition of lysis by the excess of gelatin and agar (38, 69, 175).

[31] Somewhat similar changes were postulated recently by Armstrong in the case of excitation of plant cells (5).

[32] Some of the experiments on artificial fertilization of eggs suggest that the agents which increase the activity of a cell may also cause an increase in the permeability of the cell membrane, thus permitting the escape of some of the intracellular constituents into the medium (153). Lillie has found that such cells become more permeable to water (149).

[33] It has been recognized for a long time that metabolic products of bacteria are toxic for homologous or closely related species of bacteria (75, 148). More recently, Osterhout (173) has shown that also unchanged intracellular contents may be highly toxic when applied to homologous cells from without. These effects, as ordinarily observed, are depressing in character, whereas in the case of bacteriophage, the effect is that of stimulation. This difference in effect may be due to the difference in the nature of substances concerned, but it is conceivable that it may simply depend on the concentration of injurious agent (Arndt-Shulz rule) (33, 34).

[34] The attention of the writer has been called recently to a very interesting paper of Sanfelice, who, in 1914, explained the mechanism of transmissibility of epithelioma contagiosum of pigeons on an essentially similar basis. He reported that he was able to extract from the involved areas of the skin a nucleoprotein-like substance which was regenerated anew when injected into the epithelial cells of the skin of normal birds, and perpetuated the disease in series as if it were a living agent (195).

The Nature of Resistance to Phage

One of the most interesting, yet comparatively little understood, phenomena connected with the lysis of bacteria by phage is the development of resistant cultures, which takes place *in vivo* as well as *in vitro* (65, 101, 138, 205). When susceptible bacteria are grown in broth in the presence of bacteriophage, multiplication of the bacteria is at first noticeably accelerated (67, 68, 104, 192, 197). Later, however, these bacteria undergo more or less rapid lysis, and the initial turbidity of the culture disappears. In exceptional cases the culture may be completely and permanently cleared and rendered sterile (123—p. 182). In the majority of cases, however, a certain number of bacteria fail to undergo lysis and continue to multiply, in spite of the presence of a high concentration of the lytic agent in the medium. This more or less profuse secondary growth consists of bacteria resistant to the action of bacteriophage. Such secondary cultures ordinarily lose their resistance to phage in a short time, particularly if they are transferred repeatedly to new media in order to eliminate all traces of phage by dilution, or if they are grown in media to which has been added some specific serum in order to eliminate the phage by neutralization (29, 32, 37, 41, 76, 192). Often this loss of resistance takes place slowly, and in its early stages may remain unobserved unless special effort is made to bring it to light.[35] Occasionally this property of resisting the action of bacteriophage may be retained by secondary cultures through very long periods of cultivation in the absence of phage (37, 41).

According to d'Herelle's conception, the appearance of resistant individuals in the susceptible culture is the result of specific adaptation of individual bacteria to the environment. Some cultures, according to him, may exhibit a "natural resistance" to certain races of bacteriophage, although they may be lysed by others (121—p. 259). Other cultures, on the contrary, may be so highly susceptible that all the bacteria are killed by the bacteriophage before they are able to develop any resistance (13, 123—p. 182). Ordinarily, many bacteria in a susceptible culture resist the invasion and "may thus acquire an immunity to the bacteriophage corpuscles which attack them." The phenomena of immunity in bacteria against phage are "strictly comparable with those

[35] Failure to recognize this partial return of susceptibility is undoubtedly responsible for the erroneous conclusions of several investigators who believe that the concentration of bacteriophage in the solution may increase at the expense of resistant bacteria (147, 162, 224).

observed in higher animals" (121—p. 259). According to Asheshov, this development of resistance is subject to fluctuations entirely analogous to those observed in epidemics among higher animals (8). When conditions are suitable, a certain number of bacteria "recover" from infection by directly destroying the bacteriophage corpuscles within their protoplasm (123—p. 239), or by secreting substances which inhibit the activity of the bacteriophage (101, 123—p. 240). Apart from the fact that the very possibility of the development of an active immunity by the bacteria has been doubted by many, the observation that bacteria resistant to one strain of phage may be susceptible to lysis by another homologous strain (17, 53, 99, 126, 159, 161)[36] indicates that the phenomenon cannot be explained satisfactorily upon the basis of d'Herelle's conceptions.

Furthermore, the peculiarities differentiating the secondary from the primary cultures are not limited to the former's resistance to phage. The differences in the behavior of secondary cultures are usually so numerous and often so marked as to complicate identification of the species (101, 203). There seems to be no analogy between these changes and those observed in animals during the process of immunization. For instance, resistant bacteria may differ from the organisms in the original cultures in the type of colony formed (4, 72, 93, 112, 154), in morphology, in size (58, 112, 114, 138, 154), and in motility. Many authors (29, 81) have observed that resistant forms may possess abnormal cultural characteristics, as evidenced by a marked deviation in their power to ferment carbohydrates (81, 98, 103, 170). Although the changes in serological properties are not general (41, 56, 81), and sometimes variable (50, 98), in many instances the serologic identification of the resistant bacteria is rendered difficult on account of the loss of agglutinability (112, 128, 130, 138, 157). Very striking transformations in secondary cultures have been observed also in connection with general viability (81, 115, 125), thermoresistance, and particularly in connection with virulence (to animals) which is often increased (26, 72, 138), though sometimes diminished or lost (23, 41, 82). In some instances virulence is only temporarily lost and is later regained in the absence of phage as the cultures recover susceptibility to lysis during prolonged cultivation (41).

Although it is evident that the presence of bacteriophage may be re-

[36] Although d'Herelle admits that this is true exceptionally, in general he claims that "a bacterium which once became refractory to the action of one race of bacteriophage is refractory to the action of all other races" (123—p. 239).

sponsible for the bacterial transformations described above, it is difficult to perceive how such changes could have occurred as a direct result of the development of an active immunity by the bacteria. Moreover, similar transformations of normal bacteria, including the development of resistance to phage, have been observed in instances where the organisms were not exposed to phage. Thus, for example, Hoder (127, 130) has been able to produce such a transformation by the use of various chemicals, and d'Herelle's own experiments show that the appearance of resistant forms is greatly influenced by the hydrogen ion concentration of the medium or by temperature, even in the presence of phage (123—p. 182).[37] Furthermore, similar aberrant types of bacteria have been isolated directly from normal cultures without the intervention of any agent, thus indicating that organisms potentially resistant to phage normally exist in bacterial cultures (3, 4, 96, 99).

The explanations offered thus far are either highly hypothetical (20) and not susceptible to experimental investigation, or are invalidated by the existing experimental evidence. For instance, although it is true that old bacteria are generally more resistant to phage than are younger bacteria of the same species, Marshall's (158) explanation in regard to the phenomenon of specific resistance of secondary cultures on the basis that these cultures represent the individuals which aged before they underwent complete lysis seems inadequate, inasmuch as very young forms in subcultures from the secondary growths are also resistant to lysis. Furthermore, it is difficult to explain, on this basis, why a given secondary growth resists the action of the bacteriophage which caused its selection, but is susceptible to another closely related phage.

Equally futile is the attempt to ascribe resistance in general to the production of slime (mucus) by the bacteria. Although some cultures under the influence of the active agent give rise to slime-producing variants which resist the action of phage, and although the production of slime under these conditions has been considered as a specific protective action on the part of bacteria (101), the evidence that the formation of this material is specifically related to a resistance to the phage is inadequate, inasmuch as the mucoid variants are indiscriminately resistant to all lytic agents (139). This fact and the evidence that the removal of the slime (by bile, for instance) renders the clean bacteria

[37] These findings are of particular interest in connection with the conclusion of Gratia, concerning the effects of hydrogen ion concentration on the rate of bacterial dissociation (95).

both agglutinable and susceptible to lysis (206) seem to indicate that this type of variant differs from the usual resistant forms, and is resistant merely because it is protected mechanically by a coat of slime. Furthermore, since often but few individuals among resistants arising under the influence of phage in a given culture are characterized by slime production, the mechanism of resistance to the active agent in general cannot be explained upon the basis of this phenomenon.

All these findings may be explained if one considers that the majority of individuals in a bacterial culture approaches a certain type, but that a few individuals may occasionally lack some characteristics, or may possess them in an exaggerated degree. Depending upon the conditions, some of the variants may find themselves favored by the environment, and may become quantitatively dominant. On the contrary, if the environment is changed so that it becomes incompatible with the normal development of certain types of variants, these types are eliminated more or less completely, and the predominant character of the bacterial population in the culture deviates accordingly, with corresponding changes in the biologic activity of the culture as a whole. The fact that the variants resistant to phage may be obtained from normal cultures by causing them to dissociate under the influence of heat (127), or of chemicals (95, 127, 130) seems to suggest that these variants are not the products of specific adaptation to phage, but represent the individuals in the population of the culture which survive on account of their relative tolerance toward deleterious environmental influences.

This simple explanation of the phenomenon of resistance does not, however, explain the variations in susceptibility of resistant strains to different closely related phages. Furthermore, it seems that this phenomenon cannot be explained until it is known what forces or properties are directly responsible for the higher degree of resistance of certain individuals in a bacterial population to injurious agents in general and to phage in particular, and until the specific differences in the phages themselves can be detected by other methods than by the lysis of bacteria.

Significance of Bacteriophage in Infectious Diseases

Numerous investigators have shown that the distribution of bacteriophage in nature is as wide as that of bacteria. Furthermore, it is universally agreed that bacteriophage removed from its natural environment causes the rapid dissolution of bacteria *in vitro* under suitable conditions. The question arises, therefore, as to what effect the phage

has upon bacteria in their natural environment, *e.g.*, in the intestinal canal of animals, or in polluted water and soil. It is evident that if the bacteriophage behaves in the human body as it does in a test tube, it must greatly influence the course of many infections. Indeed, according to d'Herelle, Hauduroy, Philibert, and others, the course of infection is actually determined by the presence and the ready adaptability of the phage. Furthermore, d'Herelle states that "phagotherapy" is a specific cure for bacillary dysentery and staphylococcus infections. The results of these workers in regard to infections and treatment have not been confirmed in many instances, and when claims have been made concerning the success of "phagotherapy" the procedures have not been properly controlled.[38]

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[38] This question is discussed somewhat more fully in the chapter on bacteriophage in Jordan and Falk's "The newer knowledge of bacteriology and immunology," Chicago University Press.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Lysis on agar, showing a zone of diffusion of bacteriophage around each original plaque. × 18.

Fig. 2. Early effects of phage on *B. coli*. Simultaneous presence of normal bacteria, threads and swollen bacteria. Carbol-fuchsin, followed by methylene blue. Photographed through W. W. K.$_2$ yellow filter. × 900.

Fig. 3. Moderately advanced stage of bacteriophagy. Almost all bacteria present exhibit various degrees of swelling. Threads as well as single bacteria are swollen (see also Fig. 4). Stained as above. Photographed through W. W. B. green filter. × 900.

Fig. 4. The ectoplasm of swollen bacteria, showing no visible indication of having been punctured by entering parasites. Cytoplasm showing signs of disintegration. Stained with carbol-methylene blue, followed by fuchsin. Orange W. W. E. filter. × 900.

Plate 2

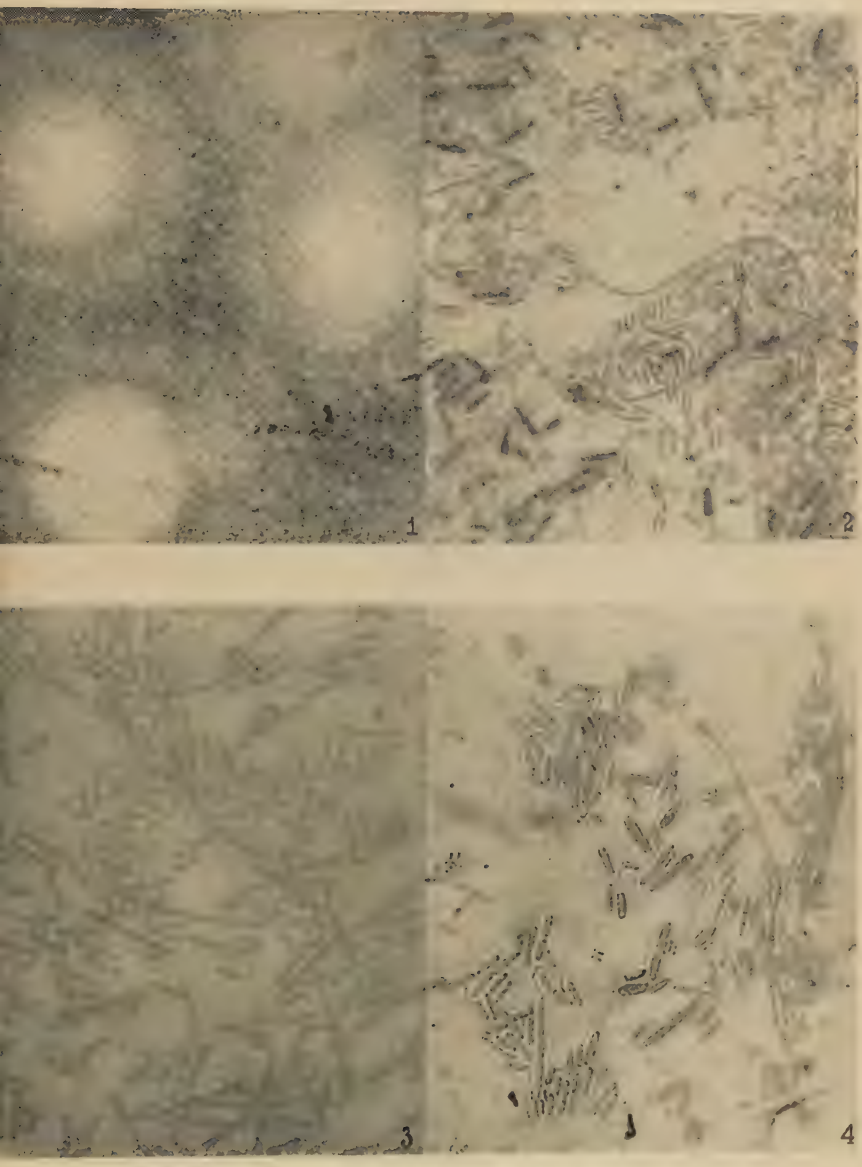
Fig. 5. Section of a cinematographic record, showing disappearance of a moderately swollen cell, indicated by the arrow in the first exposure. × 1000.

Fig. 6. Same as above, showing simultaneous disappearance of two (joined) slightly swollen cells. × 1000.

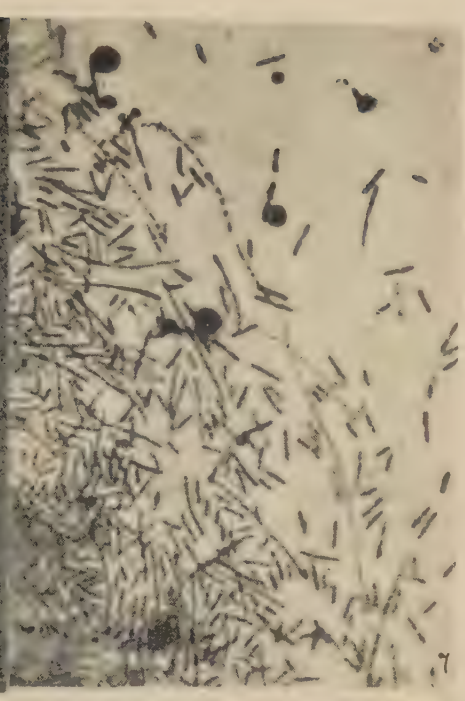
Fig. 7. Beaded appearance of the cytoplasm of threads. Stained with carbol-brilliant green, followed by fuchsin. Photographed through W. W. B. green filter. × 1000.

Fig. 8. Same as above further advanced. Stained with carbol-fuchsin, followed by methylene blue. Photographed through W. W. E. orange filter. × 1000.

PLATE 1



5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INDEX

Acidoids, 64
Adaptation of bacteriophage, to injurious agents, 386
  of viruses to new hosts, 18, 238, 343
Adsorption of acid-fast bacteria, 65
  of bacteriophage, 377
  of foot-and-mouth disease virus, 212
  of Gram-negative bacteria, 65
  of Gram-positive bacteria, 65
African horse sickness, 47
Agalactia, 43
Age of insects affected by virus diseases, 313
Aging, effect on bacteriophage, 378
  effect on viruses, 170, 214, 239, 378
Alastrim, 19, 32
  relation to smallpox, 20
  relation to varicella, 20
  relation to varioloid, 20
Alloiophylly of *Anemone nemorosa*, 341
Aluminum hydroxide, 66, 68
Aphids, 350–354
Australian X-disease, 38
Avian diphtheria, cultivation of etiological agent of, 6, 12, 256

Bacillus prodigiosus, filtration of, 71
Bacteria, effect on virus diseases, 23
  filterable forms of, 26
  filterable stages of, in relation to viruses, 22
Bacteriophage, 14
  adaptation to injurious agents, 386
  adsorption of, 377
  antibacteriophage sera, 383
  antigenic properties of, 382–383
  distribution in nature, 375–376
  filtration of, 74, 376–377
  mechanism of clearing of cultures by, 392–398
  metabolism of, 383–385
  multiplication in presence of dead bacteria, 384
  oneness of, 387–388
  organized nature of, 380–382
  origin of, 390–392
  races of, 387
  relation of lysis to production of, 388–389
  resistance to physical and chemical agents, 378–379
  respiration of, 18, 384–385
  sedimentation of, 376
  size of, 11, 377
  stimulating effect on bacteria, 396
  therapeutic importance of, 402–403
  ultrafiltration of, 376–377
Bacteriophagum intestinale, 374–375
Bacteriophagy, cinematographic record of, 396
  discovery of, 373–375
  hydrolysis during, 397
  nature of resistance to, 399–402
  pathology of, 394–398
  secondary cultures in, 399
  slime or mucus production in, 401
  stages of, 398
  swelling of bacteria in, 395
Bacterium pneumosintes filtration of, 71
Basoids, 64
Benda bodies, 246
Bile, effect on viruses, 17
Blastomycetes, 245
Bollinger bodies, 16
Borna disease, 40
  inclusions in, 134
Borrel bodies, 247, 251
Borrellina, 307
Botrytis, in aster yellows, 339

Carp-pox, inclusions in, 138
Carriers of foot-and-mouth disease, 218–220
  of poliomyelitis, 183–184
Caryoryctes cytoryctoides, 134
Cataphoresis, 66

Caterpillars, classification of blood cells in, 312
Cattle plague, 30
filterability of virus, 236
Chemicals, effect on viruses, 17, 170, 210, 255–256, 287–288, 320, 343, 379
Chlamydozoa, 1, 114, 144, 307
Chlamydozoa, 264
bombycis, 306
prowazeki, 306
Chlamydozoön myxomae, 126
Chlorosis, in virus diseases of plants, 338–339
Chromatolysis, 131, 140
Clay, 61, 67
Coccidia, 148, 245
Cold, effect on bacteriophage, 378–379
Colds, common, 46
Collargol, particle size of, 76
Contagious epithelioma, *see* fowl-pox
Contagium vivum fluidum, 19, 215
Cow-pox, 32
Cucumber, mosaic of, in relation to tobacco mosaic, 19
mosaic of, in relation to tomato mosaic, 343–344
Cultivation of fowl-pox virus, 256
of plant viruses, 354
of Rous virus, 21
of vaccine virus, 121
of viruses, 12–13
of viruses in tissue cultures, 25, 97
Cytorrhyctes aphtarum, 207
Cytoryctes vacciniae, 116
variolae, 134

Dengue fever 7, 47
Diphtheria, avian, 6, 12, 51, 256
Distemper of dogs, 41
of dogs, inclusions in, 139–140
Drying, effect on viruses, 17, 168, 170, 213, 239, 255, 287, 320, 378
Dyes, adsorption by filters, 62–64

Egg albumen, filtration of, 66
size of molecule of, 11
Electrodialysis, 61
Electroendosmosis, 62
Electroultrafilters, 61
Elementary bodies (corpuscles), 115
Encephalitis, epidemic, inclusions in, 139
following infectious diseases, 20
in Japan, 38
Koritschoner, 38
lethargic, 36
postvaccinal, 37
Epidemiology of fowl-pox, 237–239
of poliomyelitis, 180–185
Epithelioma contagiosum, *see* fowl-pox
Epitheliomyces, 245
Epithelium, cultivation of, 99
Epizoötic of guinea pigs, 30

Fettsucht of silkworm, 301
Fibroblasts, cultivation of, 99
Filterability *see* filtration
Filterable virus diseases, effect of bacterial diseases on, 23, 259, 339
effect of other diseases on, 23, 26
epidemiology of, 7
immunity to, 7
of insects, 279
list of, 4–5
of plants, affecting fruits, 341
of plants, etiology of, 354–358
of plants, immunity to, 341–342
of plants, interspecific transmission experiments on, 344
of plants, methods of control, 337
of plants, modes of spread, 337
of plants, nature of, 337–338
of plants, recovery from, 336
of plants, symptoms of, 338–343
of plants, seed transmission of, 350
of plants, transmission of, 349–354
prevention of, 10, 23, 337
Filterable viruses, biological variations of, 18–19, 252–253, 343–344
combined effect of, on plants, 339
corpuscular nature of, 18
cultivation of, 12–13, 22, 97, 121, 173, 206, 256, 354
cytotropic phenomena, 14–15, 253–255

Filterable viruses, definition of, 6
development in growing tissues, 14, 340
effect of injury on localization of, 15, 254
effect of mixtures of, 26, 220, 343
effect on cells, 15, 113, 265
effect on each other when acting simultaneously, 23, 26, 220
filtration of, 6, 10, 73–74, 171, 211, 236, 315
identity of epitheliotropic and neurotropic viruses, 19–21, 262–263
importance of young cells in reproduction of, 14, 340
inclusion bodies produced by, 15–17, 113, 244–251, 303–305, 354–358
malignant growths caused by, 21–22, 27
movement through plants, 357
mutation of, 18–19, 343–344
persistence in recovered or immune animals, 8, 9, 219, 239, 261–262, 318–319
of plants, filterability of, 338
of plants, insect-borne, 350–354
of plants, transmission by grafting, 349
question of living nature of, 19
resistance to physical and chemical agents, 17–18
respiration of, 18, 215, 384
size of, 11, 74, 212, 377
species specificity of, 13–14, 179, 316–318
technique of inoculating tissue cultures with, 99–100
Filters, Allen, 56
asbestos (Seitz, Manteufel, Uhlenhuth), 58–59
Berkefeld, 56–58
Berkefeld, tabulation of pore sizes of, 78
Chamberland-Pasteur, 55–56
Chamberland, tabulation of pore sizes of, 79
cleaning of, 84–85
composition of, 61
Coors, 56
Doulton, 56
electric charge of, 61–62
electrochemical properties of, 61–62
estimation of pore size of, 75–81
estimation of pore size by air-pressure method, 75–79
estimation of pore size by Bechhold's equation, 75
Garros, 56
inactivation of viruses by, 10
kieselguhr, 56
Kitasato, 56
Maassen, 56
Mandler, 58
Mandler, tabulation of pore sizes of, 78–79
plaster of Paris (Kramer), 59
porcelain, 55
preparation and arrangement of, 82–83
Pukall, 56
relative merits of, 60
sterilization of, 81–82
testing of, 81
Filtration, 10
adsorption during, 61–73
adsorption of antibodies during, 68
adsorption of bacteria during, 65
adsorption of complement during, 68
adsorption of dyes during, 62–64
adsorption of enzymes during, 66–67
adsorption of proteins during, 64–66
adsorption of toxins and antitoxins during, 67–68
adsorption of viruses during, 68–69
adsorptive properties of used filters, 69
of bacteriophage, 376–377
control organisms in, 82
effect of physical factors on, 83–84
of filterable viruses, 6, 10, 68, 73–74
of plant viruses, 337–338, 349, 358
relation to pleomorphism, 72–73
size of particles indicated by, 69–81
technique of, 81–85
of visible organisms, 71–73
Fish, virus diseases of, 43

Flacherie, 301
Flood fever of Japan, 51
Foot-and-mouth disease, active immunity to, 223
adsorption of virus of, 212
antibodies in immune serum of, 224
carriers of, 218–220
centrifugation of virus of, 210
compared with vesicular stomatitis, 224–226
cultivation of virus of, 206–209
discovery of ultramicroscopic nature of virus of, 206
electric charge of virus of, 211
filterability of virus of, 74, 211–212
in guinea pigs, 216–217
hyperimmunization to, 223–224
immunity to, 221–224
inclusions in, 220–221
infectiousness of virus of, 209–210
passive immunization to, 223–224
pathology of, 220–221
plurality of types of virus of, 215
properties of virus of, 209–215
resistance of virus to chemicals, 210–211
respiration of virus of, 215
serum therapy of, 223–224
size of virus of, 212
susceptibility of man and animals to, 215–218
viability of virus of, 213–214
virus of, mixed with vaccine virus, 220
Fowl diphtheria, *see* avian diphtheria
Fowl leukemia, 15
Fowl plague, 40
cultivation of virus of, 12
inclusions in, 140
filterability of virus of, 236
Fowl-pox, 12, 14, 42, 235
biological variations in virus of, 252–253
carriers of, 239
clinical picture of spontaneous disease, 239–241
comparison of disease in hens and pigeons, 238
cultivation of virus of, 12, 237, 256
distribution of virus in infected animals, 241–242
epidemiology of, 237
filterability of virus of, 236, 251–252
history of, 235–237
immunity to, 259–261
inclusions in, 244–251
incubation period of, 239
inoculated disease, 242–243
methods of staining inclusions of, 249
pathogenicity of virus for mammals, 253
pathological anatomy of, 244–251
persistence of virus in recovered animals, 9, 239, 261–262
reactions for fat in inclusions of, 246
relation to avian diphtheria, 237, 257–259
relation to mammalian pox, 262
relation to molluscum contagiosum, 244, 263
relation to variola and vaccinia, 18–19, 236–237, 262
resistance of virus to chemical agents, 256
resistance of virus to drying, 255
resistance of virus to heat, 255–256
resistance of virus to sunlight, 255
Freezing and thawing, effect on viruses, 17

Galls, in plants, caused by viruses, 357
Gelbsucht, of silkworm, 301, 306–307
Gibbs-Thomson principle, 65
Globoid bodies, cultivation of, 171–172
pathogenicity of, 172–173
Goat-pox, 32
Golgi apparatus, in fowl-pox, 248
in lymphocystic disease, 129
in relation to inclusions, 144
in relation to vaccine bodies, 120
Grasserie of silkworm, 301, 306–307, 308
Gregarines, 244
Grippe, 46
Growth of bacteria, in relation to action of bacteriophage, 393

Guarineri bodies, 16, 116–121
Guinea pig, paralysis of, 41
Guinea pig, salivary-gland disease of, 43

Haen membranes, 377
Heartwater, 51
Heat, effect on bacteriophage, 378
Hemoglobin, filtration of, 66
  size of molecule of, 11
Herpes, inclusions in, 130–133
Herpes febrilis, 36
Herpes virus, cultivation of, 12
  relation to vaccine virus, 20
  respiration of, 18
  size of, 11
Herpes zoster, 34
  relation to varicella, 20
Hog cholera, 30
  inclusions in, 139
Host specificity in polyhedral diseases of insects, 316–318
Hydrogen ion concentration, effect on bacteriophage, 379
  effect on virus of foot-and-mouth disease, 208
  effect on virus of poliomyelitis, 171
Hydrolysis during bacteriophagy, 397

Immunity, active, to poliomyelitis, 179
  agglutinins, 9
  complement-fixing antibodies, 9
  duration of, 7–9, 177, 221
  to foot-and-mouth disease, 221–224
  to fowl-pox, 259–262
  humoral, 9
  natural, to poliomyelitis, 179
  to plant viruses, 341
  to polyhedral diseases, 318–319
  precipitins, 9
  production by active and inactive viruses, 9, 261
  tissue, 9
  virucidal properties, 9
Inclusion bodies, 15–17
Inclusions, in Borna disease, 134
  in carp-pox, 138
  composition of, 143
  cytoplasmic, 116–130
  cytoplasmic and nuclear, 134–137
  in disease of fowls in Nigeria and Palestine, 140–141
  in distemper of dogs, 139–140
  division of, 147
  division of, in plants, 356
  of doubtful nature, 137–142
  in epidemic encephalitis, 139
  in fowl plague, 140
  in fowl-pox, 143, 248–251
  in fowl-pox, reactions for fat in, 246
  Golgi apparatus in relation to, 129, 144
  in herpes, 130–133
  in hog cholera, 139
  hypotheses concerning, 114–116
  in infectious myxomatosis of rabbits, 126–127
  list of, 113
  in lymphocystic disease of fish, 127–130
  in molluscum contagiosum, 125–126
  motility of, 147
  in mucous papillomata, 138
  nature of, 142–143
  nature of, in fowl-pox, 251
  nature of, in polyhedral diseases, 309–310
  nuclear, 16, 130–134
  in paravaccinia, 137
  in plants, 354–358
  in plants, enumeration of, 357–358
  in plants, similarity to those of animals, 358
  in psoriasis vulgaris, 138
  in rabies, 122–125
  relation of mitochondria to, 144
  in roots of plants, 340
  in salivary-gland disease of guinea pigs, 133
  in sheep-pox, 121
  in smallpox, 134
  in South African horse sickness, 142
  in vaccinia, 116–121
  in varicella, 133
  in virus diseases, 25
  in Virus III infection of rabbits, 133

Inclusions, in warts, 137
Infectious myxoma of rabbits, 13
inclusions in, 126–127
Influenza, 46
Inframicrobes, 3
Injury, effect on localization of viruses, 254
rôle in infectiousness of viruses, 14, 349
Insects, intermediary hosts in virus diseases of plants, 352–353
virus diseases of, 279

Jaundice of silkworm, 301, 306–307
Jurin's law, 75, 76, 77

Kaolin, 63, 66, 67
Kurloff bodies, 141–142

Leafhoppers, 350–354
Lentz bodies, 122–125
Leptospira icterohaemorrhagiae, filtration of, 72
icteroides, filtration of, 21, 72
Leukemia of chickens, 43
Liesegang phenomenon in mosaic diseases, 342
Light, effect on viruses, 213, 239, 255, 288, 320
ultraviolet, effect on activity of viruses, 20
Loeffleria nevermanni, 208
Lymphocystic disease of fish, inclusions in, 127–130
Lysis, relation to production of bacteriophage, 388–390
transmissible, *see* bacteriophagy
Lyssa bodies, 122–125

Macrophages, cultivation of, 99
Malignant growths, 21, 27
Measles, 7, 43
Membrane filters, 60–61
Membranes, collodion, adsorption by, 66
collodion, estimation of pore size, 76
Metabolism of bacteriophage, 383–385
Microbes, invisible, 3
Microdissection in the study of viruses, 130, 149
Microplasms, 3
Mitochondria, 309, 310
in relation to inclusions, 144, 248, 249
Molluscum contagiosum, 41
filterability of virus of, 236
inclusions in, 125–126
relation to fowl-pox, 244, 263
Mucous papillomata, inclusions in, 138
Mumps, 43
Myxomycetes, swarm spores of, 357

Nairobi disease, 47
Necrosis in virus diseases of plants, 339
Negri bodies, 16, 122–125, 236, 247, 354

Oroya fever, 6, 51
Overgrowth in virus diseases, 129
of plants, 339
Oxychromatic degeneration, 130–131

Pappataci fever, 47
Paralysis, of guinea pigs, 41
Parasitism, intimate type in virus diseases, 23
Paravaccinia, 6, 32
inclusions in, 137
Paschen bodies (corpuscles), 115
in vaccinia and variola, 247
Pernicious anemia of horses, 8, 30
Phloëm necrosis, 357
Pieris brassicae, 279
Plants, infectious chlorosis of, 7, 335
inclusions in, 354–358
mosaic diseases of, 28, 335
virus diseases of, economic importance of, 336
virus diseases of, effect of adaptation to new hosts, 343–344
virus diseases of, enumeration of, 343–348
virus diseases of, etiology of, 354–358
virus diseases of, host ranges of, 344
virus diseases of, importance of injury in, 349

Plants, virus diseases of, immunity to, 341–342
virus diseases of, incubation period of viruses in insect vectors, 352–353
virus diseases of, infectiousness of, 349
virus diseases of, methods of control, 337
virus diseases of, modes of spread, 337
virus diseases of, nature of, 337–338
virus diseases of, recovery from, 336
virus diseases of, systemic nature of, 340
virus diseases of, transmission of, 349–354
virus diseases of, transmission by grafting, 342, 349
virus diseases of, transmission through seed, 350
virus diseases of, treatment of, 342
Plasmolysis, by bacteriophage, 394
Pleuropneumonia, 6, 51, 247
Poiseuille's law, 76, 81, 84
Poliomyelitis, 8, 14, 38, 97, 159
active immunization to, 185
ascending paralytic type of, 161
bacteriology of, 171–174
blood picture in, 162–163
bulbar type of, 161
carriers of, 183–184
clinical picture of, 160–164
communicability of, 180
cultivation of globoid bodies, 171–172
cultivation of virus of, 12–13, 97, 171–174
distribution of virus in humans, 176
distribution of virus in monkeys, 175–176
epidemiology of, 180–185
etiology of, 166–179
experimental transmission of, 166–168
filterability of virus of, 171
history of, 159
immunity to, 179
insect vectors of, 182
milk-borne infection of, 182
morbidity of, 180–182
non-paralytic types of, 160
passive immunization to, 185
pathogenesis of, 177–179
pathology of, 164–166
physical examination of patient, 161–163
routes of injection of virus of, 174
quarantine measures against, 184
seasonal incidence of, 183
spastic type of, 161
spinal-fluid picture in, 162
spinal type of, 160
streptococci in, 173–174
symptomatology of, 160
symptomatology of experimental disease, 169–170
treatment of, 185–189
treatment with convalescent serum, 185–187
treatment with hyperimmune serum, 188–189
treatment with hypertonic solutions, 187–188
viability of virus of, 170–171
virus of, entrance into and exit from body, 180
Polyhedra, in blood cells of insects, 312–313
description of, 303–305
origin and nature of, 304–305, 309–310
relation to virus, 315–316
size of, 303
staining of, 304
Polyhedral diseases, 301
age of insects affected by, 313
control of 320–321
clinical picture of, 302–303
etiology of, 314–315
filterability of virus of, 315
host specificity in, 316–318
immunity to, 318–319
incubation period of, 314, 322
larvae susceptible to, 301–302
pathology of, 308–313
value in control of insect pests, 321
Protection, non-specific, against viruses, 20–21

Proteins, adsorption of, 64–66
Protista, 3
Protobe, 374
Protobios bacteriophagus, 375
Psoriasis vulgaris, inclusions in, 138

Quarantine, against poliomyelitis, 184
in control of virus diseases, 10, 219, 337

Rabies, 7, 14, 39
filterability of virus of, 236
inclusions in, 122–125
respiration of virus of, 18
Respiration, of bacteriophage, 18, 384–385
of herpetic virus, 18
of Indian lotus fruit, 18
of rabic virus, 18
of virus of foot-and-mouth disease, 215
Rickettsia diseases, 6, 103–104, 148
Rocky Mountain spotted fever, 50
cultivation of virus of, 12, 104
Rous sarcoma 13, 14, 15
cultivation of virus of, 12, 21, 105–106
size of virus of, 11

Sacbrood, appearance of dead larvae, 282–284
diagnosis of, 289–290
effect of phenol on virus of, 288
effect of sunlight on virus of, 288
effect of temperature on virus of, 287
etiology of, 285–286
histopathology of, 284–285
history of, 280
incubation period of, 291
infectiousness of virus of, 287
modes of transmission of, 288–289
prognosis of, 290
symptoms of, 281
treatment of, 290–291
Salivary-gland disease, of guinea pig, 14, 43
of guinea pig, inclusions in, 133
Sanarellia cuniculi, 127
Saponin, effect on viruses, 17
Scarlet fever, 6, 52
Sedimentation, of bacteriophage, 376
Serum, adsorption on glass, 64–65
Sheep, catarrhal fever of, 47
Sheep-pox, 32
filterability of virus of, 236
inclusions in, 121
Size, of bacteriophage, 377
of collargol particles, 76
of colloidal particles, 74
of viruses, 11, 24, 74, 212
Smallpox, 7, 8, 14, 19, 32, 262
inclusions in, 134
South African horse sickness, 8, 47
inclusions in, 142
Sp. biflexa, filtration of, 72
duttoni, filtration of, 72
elusa, filtration of, 72
obermeieri, filtration of, 71
parvum, filtration of, 71
Sporozoa, 244
Stomatitis, infectious papular, of cattle, 41
vesicular, 224–226
Stomoxys calcitrans, 182–183
Streptococci, in German measles, 13
in influenza, 13
in lethargic encephalitis, 13
in measles, 13
in poliomyelitis, 13, 173–174
Strongyloplasma avium, 264
hominis, 125
parvaaccinae, 137
Strongyloplasmen, 115
Strongyloplasms, 1
Stunting, in virus diseases of plants, 340
Swine-pox, 19, 32
Syphilis, 8, 262

Talc, 65, 67
Temperature, effect on viruses, 17, 167, 170, 213, 255–256, 287, 320, 342–343, 378
Tissue cultures, composition of media used in, 101–102
cultivation of fowl plague virus in, 104

Tissue cultures, cultivation of poliomyelitic virus in, 103
cultivation of Rocky Mountain spotted fever virus in, 104
cultivation of Rous sarcoma virus in, 104–106
cultivation of typhus fever virus in, 104
cultivation of vaccine virus in, 103, 104, 105
cultivation of vesicular stomatitis virus in, 209
flasks used in, 100–101
inoculation with viruses, 98–100
measurement of cell activity of, 102–103
nutritive fluids used in, 101–102
preparation of, 101–102
selection of tissues for, 98–99
serum used in, 102
in study of polyhedral diseases, 309
technique of, 98–103
type of plasmas used in, 101
Tobacco mosaic, cultivation of virus of, 13, 354
filtration of virus of, 74, 349
relation to cucumber mosaic, 343–344
size of virus of, 11
Todd bodies, 142
Tr. brucei, filtration of, 72
lewisi, filtration of, 72
Trachoma, 41, 114
Trench fever, 50
Tubercle bacillus, filtration of, 73
Tuberculosis, 8, 261
Tumors, phage-like nature of etiological agent of, 21–22
production of, by chemicals and embryonic tissues, 21–22
Typhoid fever, 7, 8
Typhus fever, 6, 49
cultivation of virus of, 12, 104

Ultrafilters, 60
Ultrafiltration of viruses, 74, 212, 376–377
Ultraviolet light, effect on virus of foot-and-mouth disease, 213

Vaccine bodies, 116–121, 142–143
Vaccine granules, 9, 120–121
Vaccine virus, 8–9, 14
cultivation of, 12, 104
filterability of, 6, 236
relation to herpetic virus, 20
relation to virus of smallpox, 19
Vaccinia, 32
inclusions in, 116–121
Varicella, 6, 7, 8, 14, 34
inclusions in, 133
relation to herpes zoster, 20
Verruga peruviana, 51
Vesicular stomatitis, 224–226
compared with foot-and-mouth disease, 224–226
Vibrio percolans, filtration of, 71
Virus, of contagious epithelioma, *see* fowl-pox
of foot-and-mouth disease, adsorption of, 212
of foot-and-mouth disease, centrifugation of, 210
of foot-and-mouth disease, cultivation of, 206-209
of foot-and-mouth disease, electric charge of, 211
of foot-and-mouth disease, filterability of, 211–212
of foot-and-mouth disease, infectiousness of, 209–210
of foot-and-mouth disease, mixed with vaccine virus, 220
of foot-and-mouth disease, plurality of types of, 215
of foot-and-mouth disease, properties of, 209–215
of foot-and-mouth disease, resistance to chemicals, 210–211
of foot-and-mouth disease, resistance to physical agents, 213–214
of foot-and-mouth disease, respiration of, 215
of foot-and-mouth disease, retention in recovered animals, 219
of foot-and-mouth disease, size of, 212
of foot-and-mouth disease, viability of, 213–214

Virus, of poliomyelitis, cultivation of, 171–174
of poliomyelitis, distribution in humans, 176
of poliomyelitis, distribution in monkeys, 175–176
of poliomyelitis, effect of pH on, 171
of poliomyelitis, filterability of, 171
of poliomyelitis, insect vectors of, 182
of poliomyelitis, methods of obtaining, 168–169
of poliomyelitis, persistence in nasopharynx, 182–183
of poliomyelitis, thermal deathpoint of, 170
of poliomyelitis, viability of, 170–171
of polyhedral diseases, 314–315
of polyhedral diseases, filterability of, 315
of polyhedral diseases, infectiousness of, 315
of polyhedral diseases, resistance to chemical agents, 320
of polyhedral diseases, resistance to physical agents, 320
of polyhedral diseases, transmission by eggs, 318–319
of paravaccinia, filterability of, 6
of sacbrood, effect of sunlight on, 288
of sacbrood, effect of temperature on, 287
of salivary-gland disease of guinea pig, 9
of typhus fever, filterability of, 6
of varicella, filterability of, 6
of vesicular stomatitis, 224–226
of vesicular stomatitis, cultivation in tissue cultures, 209
of vesicular stomatitis, infectiousness of, 225
Viruses, cytokinetic, 15, 146
cytolytic, 15, 146
effect of acetone on, 17
effect of alcohol on, 17
effect of chloroform on, 17
effect of drying on, 17
effect of freezing and thawing on, 17
effect of toluol on, 17
inactivation of filters by, 10
size of, 11, 24, 74, 212
ultramicroscopic, 3, 113, 205, 206
Virus III infection of rabbits, 13, 31
inclusions in, 133
Viscosity, increase of, in bacterial culture during bacteriophagy, 395–396

Warts, 15, 42
inclusions in, 137
Wilt, 301, 307–308
Wipfelkrankheit, 301, 305–306, 308

X-bodies, in plants, 356

Yellow fever, 48
Yellows of asters, 7
insect-borne, 351

Zoöglia, 247, 251
Zsigmondy membrane filters, 60, 77

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